Mechanistic comparison between spinal and trigeminal neuropathic pain

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DECLARATION

I hereby declare that the composition of this thesis and the work presented in it are entirely my own work, with the exception of some biochemical studies, which were carried out by Dr Emer Garry. Assistance and advice was kindly given by many individuals who are rightly acknowledged overleaf.

Some of the material from this thesis has been published:

Full Papers

Ninkina N, Papachroni K, Robertson DC, Schmidt O, Delaney L, O'Neill FE, Court F, Rosenthal A, Fleetwood-Walker SM, Davies AM and Buchman VL (2003). Neurons expressing the highest levels of □-synuclein areunaffected by targeted inactivation of the gene. *Molecular and Cellular Biology (in press).*

Abstracts

Moss A, Garry EM, O'Neill F, Dickinson T, Shen S, West K, Harmar AJ, Fleetwood-Walker SM (2001). Attenuation of neuropathic pain in $VPAC_2$ Receptor-null mice. Society for Neuroscience.

Moss A, Blackburn-Munro G, Garry EM, Blakemore JA, Mitchell R, Fleetwood-Walker SM (2001). The role of the ubiquitin-proteasome pathway in neuropathic pain. *BNA abstracts* **54.12**: P71.

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Contents

List of Figures

List of Tables

Abstract

Chronic neuropathic pain is resistant to classical analgesics and is characterised by allodynia, hyperalgesia and spontaneous pain. Pain associated with damage to the trigeminal nerve may be particularly persistent lasting twice as long as that in the spinal nerves, which possibly reflects differences between trigeminal and spinal mechanisms of synaptic plasticity. Neuropathic sensitisation occurs at the first synapses in the dorsal horn of the spinal cord and in the trigeminal spinal complex. The NMDA glutamate receptor plays a key role in this process. It is known to bind to adapter proteins, such as the membrane-associated guanylate kinases (MAGUKs including PSD-95, SAP-102, SAP-97, and Chapsyn-110), linking the receptor to a complex of signalling, anchoring, docking, and scaffolding proteins. One of these adapter proteins, PSD-95, has previously been shown by this laboratory to be crucial in the development of neuropathic pain in the spinal cord.

For this study, we used rodent models of chronic constriction injury of sciatic or trigeminal nerve to investigate the electrophysiological responsiveness of single neurones to mechanical stimuli. This strategy allowed comparison of the degree of sensitisation in the two areas. We also examined changes in expression of NMDA receptor subunits and MAGUK proteins.

Our results show a marked facilitation of responsiveness in thermal and mechanical behavioural reflexes in both spinal and trigeminal neuropathic pain models. Electrophysiological experiments indicated an increase in responsiveness of individual neurons to mechanical stimulation in spinal neuropathic animals but this increase was not as pronounced in trigeminal neuropathic animals. Further differences in electrophysiological response characteristics to various peripheral sensory stimuli between spinal and trigeminal neurons were shown in normal animals and following nerve injury. That is, neurons from neuropathic animals show a marked post-stimulus discharge response (PSDR). The length of discharge was an average of 8333 ± 1610 action potentials from spinal cord neurons and 46390 ± 16026 action potentials for trigeminal neurons, whilst the mean threshold force for eliciting a PSDR for spinal neurons was 2.1 fold that for trigeminal neurons.

Trigeminal neurons were also tested for responses to von Frey filaments both before and after a brief brush or cold stimulus applied to the face, ipsilateral to injury. Using

this protocol for before and after brush stimulation, 14 neurons from trigeminal neuropathic animals were tested. Of these, 7 neurons showed an increased initial response to low (4g) and high (15g) forces after the brush stimulus compared to that before (4g force: 12.2 spikes per second ± 0.9 before and 24.3 spikes per second ± 2.9 after. 15g force 19.6 spikes per second ± 5.8 before and 26.8 spikes per second ± 7.0 after. Conditioning stimuli experiments were not carried out in spinal cord preparation animals due to time constraints.

Biochemical experiments revealed that changes in expression of some NMDA receptor subunits, as well as associated MAGUK proteins, differed between spinal and trigeminal neuropathic animals, and within different regions of the trigeminal complex itself. A reduction in NR1 expression in the spinal cord ipsilateral to CCI compared with the contralateral side a mean reduction of 27%, was shown, whilst no change in NR1 expression was seen in any of the trigeminal regions investigated. Differential injury-induced changes were also seen in NMDA R-interacting proteins. PSD-95 shows no change in expression in regions of the trigeminal complex following CCI, but does increase in expression ipsilateral to nerve injury in the spinal cord, a mean increase of 140% compared to the contralateral side. The rise in NR2B subunit and PSD-95 protein expression at a time concomitant with the development of neuropathic pain behaviours is consistent with previous reports showing the necessity for an intact NR2B-PSD-95 complex for the development of neuropathic behaviours in PSD-95 mutant mice (Garry et al, 2003).

Furthermore, Chapsyn-110/PSD-93 which shows no demonstrable change in expression in the spinal cord exhibits a marked 40% decrease in ipsilateral expression in the trigeminal caudalis compared to the contralateral side following CCI.

We further investigated the potential role of proteins such as persyn (known to influence cytoskeletal network integrity) and α-synuclein (implicated in cell death), which may particularly influence the development, duration or recovery from neuropathic pain. We investigated the role of persyn and α-synuclein proteins in neuropathic pain, using two null-expression mutant mouse strains. However, no substantial differences were observed between the reflex behavioural responses of these mutant animals and wild type animals following nerve injury.

In conclusion, this study provides evidence for mechanistic differences in neuropathic sensitisation between trigeminal and spinal regions. These differences may lead to targets for improved therapeutic treatment of intractable pain states.

ABBREVIATIONS

AKAPs	A kinase anchoring proteins
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxadole proprionic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
°C	degrees celcius
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CCI	chronic constriction injury
cDNA	complementary DNA
CGRP	calcitonin gene related peptide
CNS	central nervous system
DAG	di-acyl glycerol
ddH2O	double distilled water
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
EAA	excitatory amino acid
ECL	enhanced chemi luminescence reagent
EDTA	ethylenediamine tetra acetic acid
G protein	guanyl regulatory protein
GABA	γ-aminobutyric acid
GAL	galanin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCl	hydrochloric acid
HRP	horseradish peroxidase
5-HT	5 hydroxytryptamine
Hz	hertz
IAN	inferior alveolar nerve
ION	infra-orbital nerve
i.p.	intraperitoneal
i.t.	intrathecal
i.v.	intravenous
kDa	kilodalton
l;ml	litre; mililitre
LTP	long term potentiation
MAP-kinase	mitogen activated protein kinase
min	minutes
MΩ	mega ohms
Mg^{2+}	magnesium
mGluR	metabotropic glutamate receptor
M;mM	molar; milimolar
mg;kg	mili; kilogram
mN/mm^2	force in milinewtons per unit area (square millimetre)
mRNA	messenger RNA
msec	millisecond
N	normal
Na^+	sodium
nA	nano amps
NaCl	sodium chloride

NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
nmol	nanomolar
NPY	neuropeptide Y
NRM	nucleus raphus magnus
$p \leq 0.05$	probability less than or equal to less that 0.05
PACAP	pituitary adenylate cyclase-activating polypeptide
PAG	periaqueductal grey
PBS	phosphate buffered saline
PF	paraformaldehyde
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PNL	partial nerve ligation
PSDC	post synaptic dorsal column
RNA	ribonucleic acid
s	seconds
s.c.	subcutaneous
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ser	serine
SEM	standard error of the mean
SMP	sympathetically mediated pain
SMT	spinomesencephalic tract
SOM	somatostatin
SP	substance P
SPET	suspended paw elevation time
SPN	sympathetic postganglionic neurones
SRT	spinoreticular tract
SSC	standard saline citrate buffer
STT	spinothalamic tract
thr	threonine
tRNA	transfer RNA
TTT	trigeminothalamic tract
TTX	tetrodotoxin
VIP	vasoactive intestinal polypeptide
vol	volume
wt	wild-type

Chapter 1

Introduction

General Introduction

At present we possess only an approximate definition of pain. This is largely due to the complexities of describing the emotional dimension of pain and the sometimes loose association between pain and injury. The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey, 1983). The associated difficulties in relating human and animal experience on a conscious and emotional level, have suggested that the term pain may be more accurately described by that of nociception, the physiological response of an animal to a noxious stimulus. However pain related words such as hyperalgesia and allodynia to described animal behaviours are almost unavoidable.

The ability to experience pain is protective. It alerts against potential tissue harm and also promotes guarding of damaged tissues until healed. Acute pain therefore is a normal physiological response. However, some pain conditions can be persistent and outlast both the noxious stimulus and period of healing. These conditions can last for many years and be so debilitating that quality of life is severely reduced.

Neuropathic pain, with which this thesis is concerned, is a persistent pain state that occurs as a result of nerve damage and at present is poorly treated by conventional analgesic pharmacology.

1.0 Normal Sensory Transmission

We will begin with a general description of normal somatosensory processing including an overview of the types of nerve fibre and their receptors, the anatomy of the spinal cord and the trigeminal system,

1.1 Classification of Sensory Afferent Nerve Fibres

The skin is richly supplied by specialised nerve fibres, which convey environmental information to the central nervous system. Afferent axons run uninterrupted from their sensory endings in the peripheral target tissue to their central synaptic terminals in the CNS.

The sensory nerve fibres can be classified according to their size.

They are;

1. Large myelinated Aβ fibres, (5-15μm in diameter) with conduction velocities of 30-100m/sec (cat). Many respond to light touch under normal physiological conditions (Willis and Coggeshall, 1991).

2. Smaller Aδ fibres (1-5 μm diameter, Willis and Coggeshall, 1991) with conduction velocities of 6-30m/sec (cat). These respond to light and heavy pressure, heat, noxious chemicals, and cooling).

3. Smaller unmyelinated C-fibres (0.25-1.5μm diameter, Gasser, 1950), with conduction velocities of 1-2.5m/sec (cat). The majority of these nerve fibres respond to almost all of the above mentioned stimuli and are termed polymodal fibres). The axons of these nerve fibres innervate the cutaneous skin and have specialised endings which are receptor specific, and therefore can be classified according to function.

The rat sciatic nerve originates from spinal segments L4-L6 and comprises sensory, motor, and sympathetic fibres. At the level of the mid-quadriceps the nerve contains approximately twenty-seven thousand axons of which 23% are myelinated sensory axons, 48% unmyelinated sensory axons, 23% sympathetic axons and 6% are motor axons (Schmalbruch, 1986). The composition of the trigeminal infraorbital nerve is similar to that of the sciatic nerve but with a reduction in the proportion of sympathetic fibres present (Jacquin et al, 1994).

1.2 Classification of Sensory Nerve Fibre Receptors

Each axon terminates in a specialised formation in close contact with the skin. The area of the skin which when stimulated will excite a receptor is called its receptive field. They have been identified experimentally to respond to specific stimuli (for example, heat, pressure and certain chemicals) in distinguishable classes of non-nociceptors and nociceptors (Lynn, 1994).

1.2.1 Non-nociceptive Mechanoreceptors

Cutaneous mechanoreceptors are the most sensitive receptors, responding most readily to mechanical pressure stimuli of various intensities. They can be further subdivided according to their other characteristics:

Hair follicle receptors. These receptors, as their name suggests, are found in hairy skin, their terminal endings wrap around the hair shaft and are activated by hair movement. They are the predominant class of units with myelinated Aδ axons within rat peripheral nerves (Lynn and Carpenter, 1982), and can be classified according to the type of hair which they innervate (Brown and Iggo, 1967). **D-hair** units tend to have relatively slowly conducting axons with large receptive fields, and respond well to slow movement of the fine down hairs, while **G-hair** units with their larger axons have relatively small receptive fields and tend only to be activated by fast movement of the guard hairs (Lynn and Carpenter, 1982). **T-hair** units also exist, but are the least numerous, and these units can be excited by movement of the large tylotrich hairs.

Cutaneous Receptors-Slowly Adapting (SA). These receptors will continue to be activated throughout the time a stimulus is applied. There are two types. Slowly adapting type I mechanoreceptors (SAI) are low threshold receptors associated with Merkel cell complexes in the basal layer of the epidermis. They constitute 25% of all Aβ fibres, respond to >50 microns indentation and fire continuously in the presence of a probe pressed into the skin. Slowly adapting type II mechanoreceptors (SAII) are identified with Ruffini endings located in the dermis. They constitute 19% of Aβ fibres, are responsive to >200 microns indentations and continually fire in response

to lateral stretch of the skin (Sinclair, 1982, Willis and Coggeshall, 1991). Together they detect both the displacement and velocity of mechanical stimuli, and tend to fire to maintained skin or joint displacements (Lynn and Carpenter, 1982).

Cutaneous Receptors-Rapidly Adapting (RA). These receptors are active mostly at the beginning and end of a stimulus, again there are two types. Rapidly adapting type I mechanoreceptors (RAI), 43% of all Aβ fibres, attached to Meissner corpuscles, respond to skin indentation of >10 microns, and are sensitive to low frequency vibrations. Rapidly adapting type II receptors (RAII), 13% of Aβ fibres, attached to Pacinian corpuscles and Golgi-Mazzoni receptors, respond to indentation of >8 microns and to high frequency vibration.

Low Threshold C-fibre Mechanoreceptors are sensitive mechanoreceptors with unmyelinated afferent fibres, and a distinct group of C-fibres with a high sensitivity to mechanical stimulation (Bessou and Perl, 1969). They are rapidly adapting, have a small receptive field and are mostly found on hairy skin. They constitute between 15-30% of C fibres in peripheral nerves. These receptors rarely respond to warm thermal stimuli, although a large proportion has been reported to respond to cold stimuli (Bessou and Perl, 1969; Leem et al, 1993; Lynn and Carpenter, 1982).

1.2.2 Non-Nociceptive Thermoreceptors

Non-nociceptive thermoreceptors respond to changes in temperature but not mechanical stimuli. They are separated into two types.

Cold Thermoreceptors Cool sensations are signalled by activity in cold specific Aδ-fibres (and to a lesser degree cold-specific C fibres) (Iggo, 1959; 1969), with specific cutaneous receptors in both the hairy and glabrous skin (Iggo, 1969). Cold thermoreceptors are characterised by their high sensitivity to small reductions

in skin temperature (as little as 0.1°C), and the most commonly studied in the rat are facial and scrotal thermoreceptors. The majority of these receptors have a relatively restricted range of innocuous cold temperatures (approximately 20-30°C) over which they give dynamic responses to small reductions in skin temperature (Heinz et al, 1990; Iggo, 1969).

Warm Thermoreceptors. Warm thermoreceptors respond to slight warming of the skin and are thought generally to be unmyelinated (Iggo, 1959). They are active at normal skin temperature (approximately 30°C) and are silenced by noxious levels of heat (>48°C).

1.2.3 Nociceptors

Nearly a century ago Sherrington proposed the existence of the nociceptor, a receptor that is activated by stimuli capable of causing tissue damage (Sherrington, 1906). It was only sixty years later that, electrophysiological experiments showed the existence of primary afferent neurons that were activated by noxious heat, intense pressure and irritant chemicals (Burgess and Perl, 1967). There are two main groups of cutaneous nociceptors, the A fibre nociceptor and the C-polymodal nociceptor. They innervate the skin and the viscera, ending in free nerve endings. Microneurography and intraneural micro-stimulation of identified cutaneous primary afferent fibres in humans demonstrated that electrical stimulation of nociceptive Aδ fibres evoked a fast sensation of sharp pain (first pain), whereas stimulation of nociceptive C fibres produced a slow sensation of dull or burning pain (second pain), (Torebjork and Ochoa, 1980; Ochoa and Torebjork, 1989).

Aβ Fibre Nociceptors. Aβ fibre nociceptors have been described (Burgess and Perl, 1967; Burgess et al, 1968) but are relatively under-reported today. The proportion of A fibre nociceptors with Aβ fibre conduction velocities has been claimed to be as much as 67% in the rat (Ritter and Mendell, 1992; Lawson, unpublished data), and even though they are present in the tooth pulp the

contribution they make to pain conditions is still unknown due to little subsequent investigation.

Aδ Fibre Nociceptors. There are two main classes of Aδ fibre nociceptor, both respond to intense mechanical stimuli, that can be distinguished by their responsiveness to intense heat. Aδ fibre nociceptors are primarily high threshold mechanoreceptors responding to high threshold mechanical (HTM) stimulation of the skin, such as pressure and pinch. **Type I** Aδ fibre nociceptors respond to noxious heat at 53°C or higher. **Type II** Aδ fibre nociceptors respond to heat at around 43°C. Work by Simone and Kajander, (1996; 1997) has shown that all mechanosensitive Aδ nociceptors responded to noxious cold stimuli. Leem et al, (1993) classed 70% of Aδ nociceptors as mechanical nociceptors, 20% as mechano-heat nociceptors and 10% as mechano-cold nociceptors. They exist in both glabrous and hairy skin.

C-Fibre Nociceptors. These units are the most common form of primary C-afferent unit found within peripheral nerves of the rat (Lynn and Carpenter, 1982; Schmalbruch, 1986). They respond to multiple stimulus modalities, hence the term polymodal, exist in both hairy and glabrous skin, and typically have small receptive fields. C-fibre nociceptors generally respond to heat at initial temperatures of 42°C-55°C (ranging from borderline to overtly noxious and damaging). These fibres are also excited by irritant or caustic chemical agents applied to the skin and typically respond to high threshold mechanical stimulation (Perl, 1984). The vast majority of cutaneous C-fibre nociceptors have been shown to terminate in laminae I-II of the spinal dorsal horn (Cervero and Iggo, 1980; McMahon et al, 1984).

Recently it has become clear that nociceptors may also be subdivided on biochemical differences. Some nociceptors co-express the neuropeptides calcitonin gene related peptide (CGRP) and substance P. These cells are dependant on the neurotrophin nerve growth factor (NGF) for survival, and express the NGF receptor TrkA. Another group of nociceptors do not express the TrkA receptor and instead are sensitive to glial cell-derived neurotrophic factor (GDNF). These cells also express the enzymes fluoride-resistant acid phosphatase (FRAP), the receptor tyrosine kinase Ret, as well as binding sites for the isolectin B4 (IB4) (Molliver et al., 1997). The

functional relevance of these findings is not yet known but some cells in both these subtypes seem to express the vanilloid receptor VR1, which binds capsaicin and is also activated by thermal stimuli (Caterina et al, 1997). The VR1 receptor can be co-localised with IB4 binding sites and P2X3 receptors, which indicate that these neurons are nociceptive (Guo et al, 1999). In the CGRP/SP subset, VR1 was also co-localised with SP and CGRP in cell bodies in the DRG but much less so in the terminals of these neurons in the spinal cord (Guo et al, 1999).

Finally, the natural stimulus that activates some nociceptors is difficult if not impossible to identify. These have been termed silent or sleeping nociceptors, and appear responsive only when sensitised by tissue injury (Schmidt et al, 1995).

1.3 Sensory processing in the spinal cord

1.3.1 Anatomy of the Spinal Cord

The spinal cord receives primary afferents in segments from the cervical through to the sacral levels. The cell bodies of these afferent nerves are located in the dorsal root ganglion (DRG) located to each side of the spinal column. Most afferent fibres pass through the dorsal root but some may pass via the ventral root (Coggeshall et al 1975). Each segment corresponds to a discrete region (somatome) of the body innervated by these primary afferents and together they create a somatotopic map of the body. Efferent nerves leave the spinal cord at each segment.

1.3.2 Laminar Organisation of the Dorsal Horn of the Spinal Cord

The grey matter of the spinal cord is arranged in a butterfly shape and comprises nerve cell bodies, fibres (axons and dendrites) and associated non-neuronal cells. It can be grossly divided into two main sub-divisions, the ventral (motor) horn and dorsal (sensory) horn. These regions can be further subdivided into discreet horizontal zones when seen in transverse section. These laminae were first documented by Rexed, (1952) in the cat, with the laminae being classified according to their cytoarchitectonic characteristics. Further anatomical studies have demonstrated a similar architectonic scheme in the rat (Molander et al, 1984).

According to Rexed's nomenclature, the dorsal horn of the spinal cord is sub-divided into 6 distinct laminae.

Lamina I (LI, The Marginal Zone)

The narrowest and most superficial layer of the dorsal horn (Molander et al, 1984), LI contains small medium and large neurons, receives both unmyelinated C fibres and myelinated Aδ- fibres (Willis and Coggeshall, 1991) but not large myelinated Aβ fibres (Brown, 1981). These afferents project to the thalamus, midbrain, or to other parts of the spinal cord (Willis and Coggeshall, 1991). Studies have revealed a large proportion of rat LI cells to be multireceptive (McMahon and Wall, 1983; Menetrey and Besson, 1981). Noci-specific neurons which receive their projections from cutaneous, high threshold Aδ mechanoreceptors and C fibre thermal nociceptors have also been reported (Cervero et al, 1976; 1979; Christensen and Perl, 1970; Light and Perl, 1979; Rethelyi et al, 1983), highlighting the importance of LI in the transmission of nociceptive sensory information. Neurons in lamina I have been classified morphologically in to three classes (Lima et al., 1991; Zhang et al, 1996) (1) fusiform cells with small, spindle-shaped somata and bipolar, longitudinal dendritic arbors; (2) pyramidal cells with triangular somata and three main dendritic origins with primarily longitudinal arborisation; and (3) multipolar cells with larger, multiangular somata and four or more radiating dendritic arbors directed both longitudinally and mediolaterally. The overall proportions of cell types were 34% fusiform, 36% pyramidal, 25% multipolar, and 5% unclassified. Many lamina I neurons can be retrogradely labelled from the brainstem (Menétrey et al., 1982, 1983; Cechetto et al., 1985; Hylden et al., 1989; Lima & Coimbra, 1989; Lima et al., 1991). In a previous study Todd et al, found that the largest numbers of labelled lamina I neurons were seen following tracer injections into the caudal ventrolateral medulla (CVLM) or lateral parabrachial area (LPb), while a smaller number was observed with injections into the periaqueductal grey matter (PAG) (Todd et al., 2000).

Dorsal horn neurons which express the neurokinin 1 receptor contribute to several ascending pathways that are thought to be important in pain mechanisms (Todd et al, 2000) and recently, it has been shown that lamina I projection neurons in which long

term potentiation (LTP) can be induced express both the NK1 receptor and T-type voltage gated calcium channels, both of which are necessary for LTP (Ikeda et al, 2003).

Lamina II (LII, The Substantia Gelatinosa)

Lamina II (LII), also called the 'substantia gelatinosa', lies ventral to LI and consists of an outer zone (LII_o) which contains densely packed small cells (5x5μm) and a less compact inner zone (LII_i). Again it receives a majority of unmyelinated C fibres and myelinated Aδ-fibres (Willis and Coggeshall, 1991). Aß fibres can innervate this region (Wilson and Kitchener, 1996). However, the extent of this innervation after peripheral nerve injury has not yet been precisely determined as the selective A-fibre retrograde tracer used (cholera toxin b subunit) may be transported by small afferents after nerve injury (Shehab et al, 2003). It has been suggested that sprouting of Aβ inputs may be an important yet understated factor possibly involved in neuropathic changes (See section 1.10.1). The dendrites in LII extend longitudinally through LII and LIII in the saggital plane, but are severely restricted in the transverse plane, whilst the axons of LII cells are confined for the most part within LII (Matsushita, 1969). Thus, LII contains mostly intrinsic interneurons with extensive local integration. Lamina II is concerned predominantly with the processing of sensory information from the skin and receives very little input from the non-cutaneous structures (muscle and viscera) (Wilson and Kitchener, 1996). Only a few (~1%) LII neurons project axons to the brainstem (Giesler et al, 1976; Willis et al, 1979), whilst some neurons project into LI. Nociceptive afferents, many of which contain SP, terminate mainly in laminae I and II of the spinal dorsal horn. Correspondingly, up to 80% of lamina I projection neurons express the SP receptor, NK_1 (Todd, 2002). Together LI and LII are known as the 'superficial dorsal horn'.

Laminae III – IV (LIII-LIV)

Lamina III (LIII) lays ventral to LII, is broader than both LI and LII, and contains generally larger (7-8μm x 10-12μm) and less tightly-packed cells (Rexed 1952). Intracellular injections and retrograde staining studies have demonstrated that

significant populations of lamina III neurons project to the spinocervical nucleus and into the dorsal columns (Brown, 1981; Brown et al, 1977). Lamina IV (LIV) is a thicker layer, ventral to LIII and contains large, scattered cells (10x15μm) many of which project dendrites into LI –III and are therefore able to receive a direct primary afferent input from fibres that enter the superficial layers. LIII-LIV also contains cells of origin of the spinocervical (SCT), and the postsynaptic dorsal column (PSDC) tracts (Brown and Fyffe, 1981). LIII-IV receives Aβ primary afferents from large hair follicles and SA and RA mechanoreceptors and non-nociceptive Aδ fibres (Brown and Iggo, 1967; Light and Perl, 1979).These laminae also receive input from visceral afferents (Szallasi et al, 1995; Gillette et al, 1994).

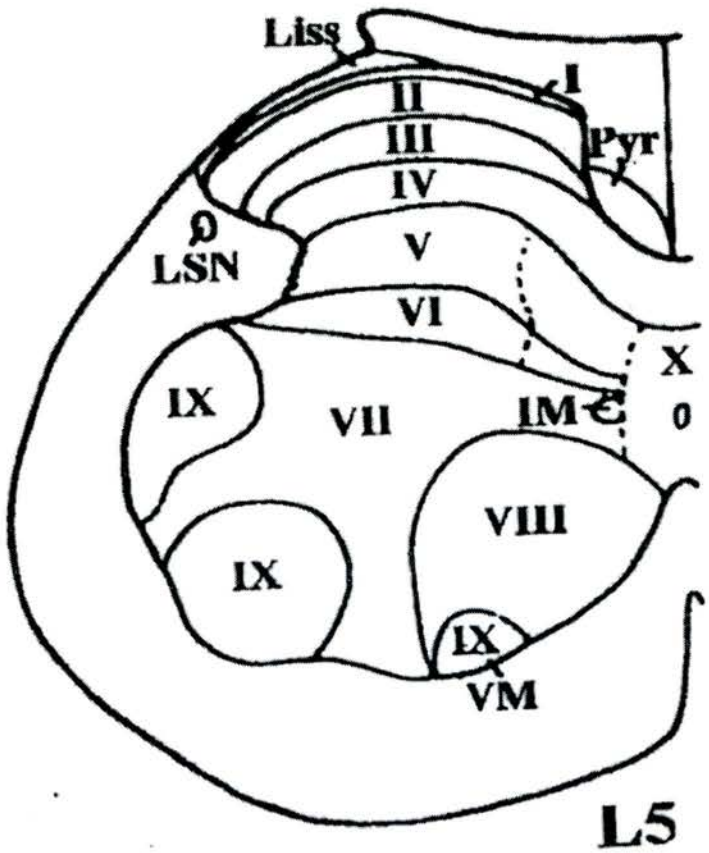
Laminae V-VI (LV-LVI)

Lamina V extends as a thick band across the narrowest part of the dorsal horn and contains many bundles of myelinated fibres as well as a large number of large diameter cells (Molander et al, 1984). Lamina VI only exists in the cervical and lumbosacral enlargements of the spinal cord where it represents the transitional layer between the primary afferent-dominated dorsal horn and the ventral horn. Very few primary afferents terminate here and this lamina is dominated by a large number of small neurons (Willis and Coggeshall, 1991).

The ventral horn contains laminae VII-X, but is beyond the scope of the present study. It is important to note that laminae are recognised primarily as zones of particular cell types and may have indistinct borders. Also the borders between the laminae may differ between different segmental sections (Molander et al, 1984).

Figure 1.1

Schematic Diagram of the Cytoarchitectonic Organisation of Segments LI-VI of the Rat Spinal Cord



Representation of the laminar divisions of Rexed (1952) as demonstrated in the lumbar (L) segments of the rat spinal cord.

Abbreviations:

I – X – spinal cord laminae; CC – column of Clarke; IL – intermedio-lateral nucleus; IM – intermedio-medial nucleus; LSN – lateral spinal nucleus; Liss – Lissauer's tract; LG – lateral group of large cells in the dorso-lateral part of the ventral horn; LM – latero-medial nucleus; MG – medial group of large neurones in the intermediate zone; Pyr – pyramidal tract; VM – ventro-medial nucleus. Note that LG, LM and VM are parts of lamina IX (Molander et al., 1984).

1.4 Sensory processing in the Trigeminal System.

The trigeminal nerve is the main nerve carrying somatosensory information from the orofacial structures. The composition of the trigeminal nerve is similar to that of the spinal nerves, but has a relatively lower proportion of sympathetic fibres present (Jacquin et al, 1994). The cell bodies of most of the primary afferents are located in the trigeminal ganglion at the base of the skull. The nerve has three branches, the ophthalmic branch, the maxillary branch and the mandibular branch. It has a distribution covering all of the face and oral cavity. It carries sensory information from the face, the mucosa of the oral cavity, the bony tissues and the teeth and the motor component of the muscles of mastication. The primary afferents enter the brainstem at the level of the pons. As they enter the brainstem different fibre types separate and enter different regions of the trigeminal system in the brainstem. These regions are divided into the mesencephalic nucleus, the principal sensory nucleus and the trigeminal spinal complex. The spinal trigeminal complex is further subdivided into three subnuclei. Descriptive studies of cell type and function in the trigeminal system are not as numerous as those for the spinal cord and comparable immunohistochemical experiments are relatively sparse. Therefore the information on cell types in this system largely consists of anatomical/morphological studies.

The Mesencephalic Nucleus

This nucleus is different from the others of the trigeminal system or equivalent spinal dorsal horn region because it contains the cell bodies of the primary afferents. It is associated with and is the only instance of primary afferent nerve cells having their cell bodies in the central nervous system. It is a proprioceptive nucleus and processes information from afferents innervating structures such as the periodontal ligament, and muscle stretch receptors (Hildebrand et al, 1995). It is located in the periaqueductal grey area and is flanked on either side by fibre tracts. This nucleus has no involvement with nociception.

The Trigeminal Complex

This refers to the combination of the principle sensory nucleus, the oralis, interpolaris and the caudalis. Olszewski originally described these as nuclei in 1950 and reserved the term subnuclei for the different layers in the regions themselves which can also be termed zones.

The Trigeminal Principal Sensory Nucleus

This nucleus receives mostly the large myelinated Aβ fibres conveying touch information from the skin and surrounding hairs. It also receives fibres from the whiskers in animals which possess them and in this case there is a specialised region which has a specific area dedicated to each individual whisker. Following single cell electrophysiological recording work, the principle sensory nucleus is not thought to have a major role in the processing of nociceptive information (Sessle, 1999).

The Trigeminal nucleus oralis (SpO)

Situated inferior to the principal sensory nucleus, the trigeminal nucleus oralis consists of only one morphological cell type (12-25μm in diameter). These cells are triangular or spindle shaped, slender and densely packed. This region has been known to be involved in nociception for some time (Wall and Taub, 1961). More recent studies have shown that the application of mustard oil (MO) to the tooth pulp in rats induces prolonged NMDA receptor-dependant neuroplastic changes with enhancement of SpO neuronal receptive fields and response properties (Park et al, 2001), and that this process is also dependant on the integrity of the more caudal trigeminal nucleus caudalis (Chiang, et al 2002).

The Trigeminal nucleus interpolaris (SpI)

This structure is uniform and consists of two morphological cell types; small diameter cells (12-25μm), which are oval or triangular in shape and larger diameter cells (25-40μm) which are round or oval and have very short dendrites (Olszewski, 1950). Surprisingly no changes were found in most of the axo-somatic synapses following trigeminal rhizotomy (Westrum and Black, 1968). It is thought that this nucleus may

contribute primarily to inputs to the cerebellum rather than the thalamus, however, localised lesions in the interpolaris have also demonstrated long axon projections to the reticular formation (Carpenter and Hanna, 1961). It has been shown that the lateral portion of the SpI extends caudally into the region of the trigeminal nucleus caudalis adjacent and caudal to it, creating a SpI/SpO transition region which seems to be an important area for integration of peri-oral and intra-oral sensory input. (Phelan and Falls, 1989; Sessle 2000). Also, the SpI has been shown to respond to tooth pulp stimulation in awake cats (Boissonade and Matthews, 1993).

The Trigeminal nucleus caudalis (SpC)

The structure of the trigeminal nucleus caudalis is thought to be much more akin to that of the spinal dorsal horn. It can be divided into three zones. The most lateral zone is the zona marginalis, it is a thin band populated by a few large (20-30μm in diameter) dark multipolar cells with long dendrites and is homologous with lamina I of the dorsal horn. Lying medio-dorsal to the zona marginalis is the zona gelatinosa, which has the shape of a horseshoe, is composed of small densely packed oval cells (7-20μm in diameter), it is believed to be equivalent to laminae II-III of the dorsal horn. The most medial zone is the zona magnocellularis, it contains slender multipolar cells of varying sizes with long dendrites and is homologous with the deeper laminae of the dorsal horn IV-VI (Olszewski, 1950).

There are many reports over the last 70 years that disruption of the trigeminal nucleus caudalis can interfere with facial pain and nociceptive behaviour in humans and in animals. These have included clinical observations of the effects of trigeminal tractotomy, the neurosurgical procedure which has sometimes been used for the relief of trigeminal neuralgia. The operation involves transection of the trigeminal tract usually at the rostral border of the nucleus caudalis and can reduce the patients' ability to perceive noxious stimuli to the face with a limited loss of tactile sensitivity (Gerard, 1923; Sjoqvist, 1938). These clinical reports are consistent with experimental studies in animals which show that disruption of the nucleus caudalis, either surgically or chemically, can interfere with an animal's apparent perception of a noxious stimulus to the face (Duale et al, 1996; Denny-Brown and Yanagisawa, 1973; Sessle, 1987, and 1996).

There has been considerable evidence for similarities between the spinal dorsal horn and the trigeminal nucleus caudalis (Dubner and Bennett, 1983). The spinal dorsal horn is continuous with the trigeminal nucleus caudalis and the reticular formation (Gobel et al, 1977), and the locations of trigeminothalamic and spinothalmic neurons in laminae in both areas are similar. Also, orthograde and retrograde tracer studies have identified similar descending anatomical and neurochemical pathways in the spinal dorsal horn and its trigeminal homolog, and eight Laminae similar to laminae I-VIII of the spinal cord have been identified on an anatomical basis. It has been suggested that the trigeminal nucleus caudalis together with the adjacent reticular formation should be termed "the medullary dorsal horn" (Gobel et al 1981). This term will be used in some places in this text. Its use does not imply that no differences exist at medullary and spinal levels but, that differences may be no greater than those seen between distant spinal segments and it serves to differentiate the caudalis part of the trigeminal complex from the anatomically distinct oralis and interpolaris. However whilst there are similarities with the dorsal horn of the spinal cord, there are also distinguishing factors that differentiate these two regions. Firstly, the trigeminal complex also receives convergent nociceptive information from other cranial nerves, the facial, glossopharyngeal and vagus nerves as well as the first two branches of the cervical nerves (Sessle, 1996) although some parallel may be drawn from the innervation of spinal cord from different segments. Secondly, the trigeminal complex shows features of redundancy/parallel processing in that several regions receive similar information from the same source. Studies that have documented the involvement of the rostral components of the trigeminal nuclear complex, including trigeminal nucleus oralis and interpolaris in orofacial pain mechanisms (Wall and Taub, 1961; Duale et al, 2001; Dallel et a, 1999; Parada et al, 1997; Park et al, 2001; Boissonade and Matthews, 1993) are substantiated by lesion studies that show damage to the caudalis region does not completely abolish nociceptive responses evoked by noxious intra-oral or peri-oral stimuli, and disruption of rostral components including the oralis has been reported to interfere with nociceptive orofacial sensation or behaviour (for review, see Sessle 2000). The application of the inflammatory irritant, mustard oil (MO), to the tooth pulp can produce neuroplastic changes characteristic of central sensitization in nociceptive neurons of oralis and caudalis (Chiang et al. 1998; Park et al. 2001), but it has been shown recently that inflammatory pulp-induced central sensitization in oralis is dependent on the

functional integrity of caudalis (Chiang et al, 2002). In fact it has long been known that very few C-fibres project into the oralis subnucleus while many project to the substantia gelatinosa of the caudalis (Windle, 1927), and electrophysiological studies confirming dependence of C-fibre activity in the trigeminal nucleus oralis on the integrity of caudalis substantia gelatinosa helped explain why latencies of C-fibre responses were longer in the oralis than caudalis even though they had less distance to travel from the stimulating electrode (Pajot et al, 2000). These last experiments have suggested a model where the majority of C-fibres in the trigeminal peripheral nerve enter the trigeminal complex at the level of the trigeminal nucleus caudalis and pass information on to the oralis via connections in the caudalis substantia gelatinosa.

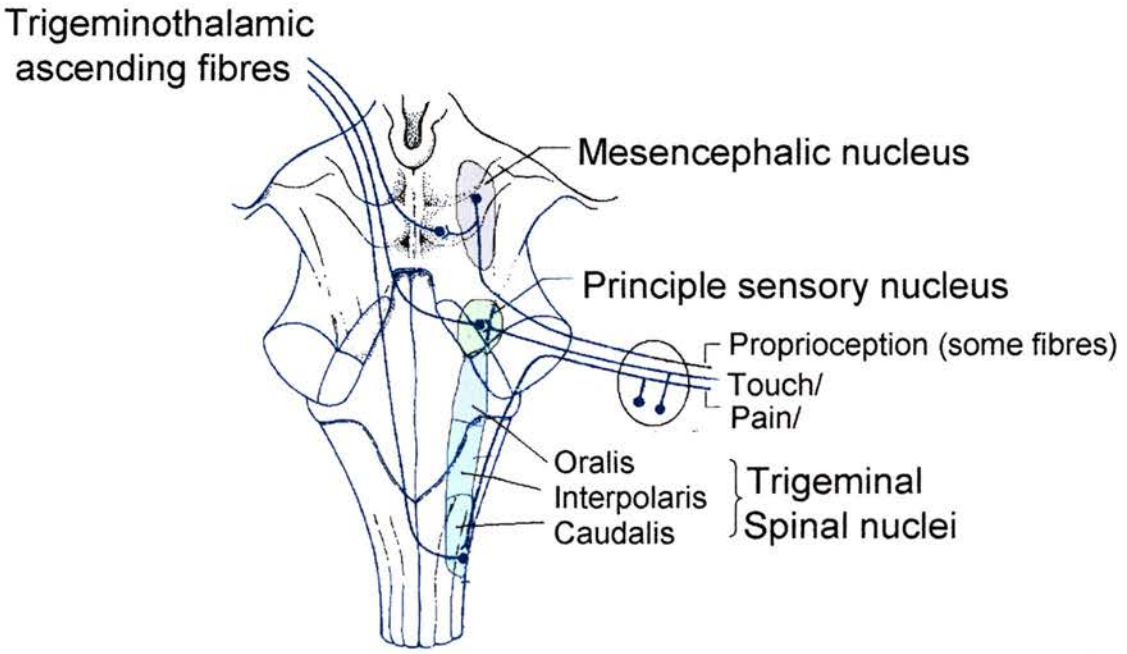
In spinal dorsal horn substance P- and CGRP-positive fibres project mainly to lamina I and outer lamina II, regions of sparse IB4 immunoreactivity, while IB4- positive fibres project mainly to inner lamina II (Snider and McMahon, 1998; Basbaum, 1999; Woodbury et al., 2000). Although the exact relationship to pain processing remains uncertain, the distinctive patterns of IB4-negative and IB4-positive fibres in the spinal dorsal horn suggest different roles in nociception. Compared to spinal dorsal horn the caudal SpC displays a broader density of IB4-positive fibres which crosses laminae I, IIo, and IIi (Ambalavanar and Morris, 1992; Sugimoto et al., 1997a) and overlaps that for substance P and CGRP (Ambalavanar and Morris, 1992; Sugimoto et al., 1997b). The wider distribution of IB4 positive fibres in the superficial laminae of caudal SpC compared to spinal dorsal horn may be related to the extensive convergence from cervical rootlets and multiple cranial nerves received by the caudal SpC (Renehan and Jacquin, 1993; Sessle, 2000). By contrast, the ventral SpI/SpC transition region displays dense immunoreactivity for substance P and CGRP and lacks staining for IB4. These results suggest that the ventral SpI/SpC transition region and caudal SpC receive a different complement of unmyelinated C-fibres.

It is also interesting to note that there is a difference in embryological development of the cells that form the dorsal root ganglion and therefore the peripheral sensory nerves and cells that form the trigeminal ganglion and therefore some of the trigeminal sensory nerves. All sciatic DRG afferents derive from neural crest cells, while many of the trigeminal ganglion afferents are derived from ectodermal placode cells as well

as neural crest (D'Amico-Martel and Noden, 1983). It is known that neural crest cells and ectodermal placode cells differ in physiological characteristics (Ebenal and Hedlund, 1975; Le Douarin, 1983) but none so far have been related to pain.

Figure 1.2

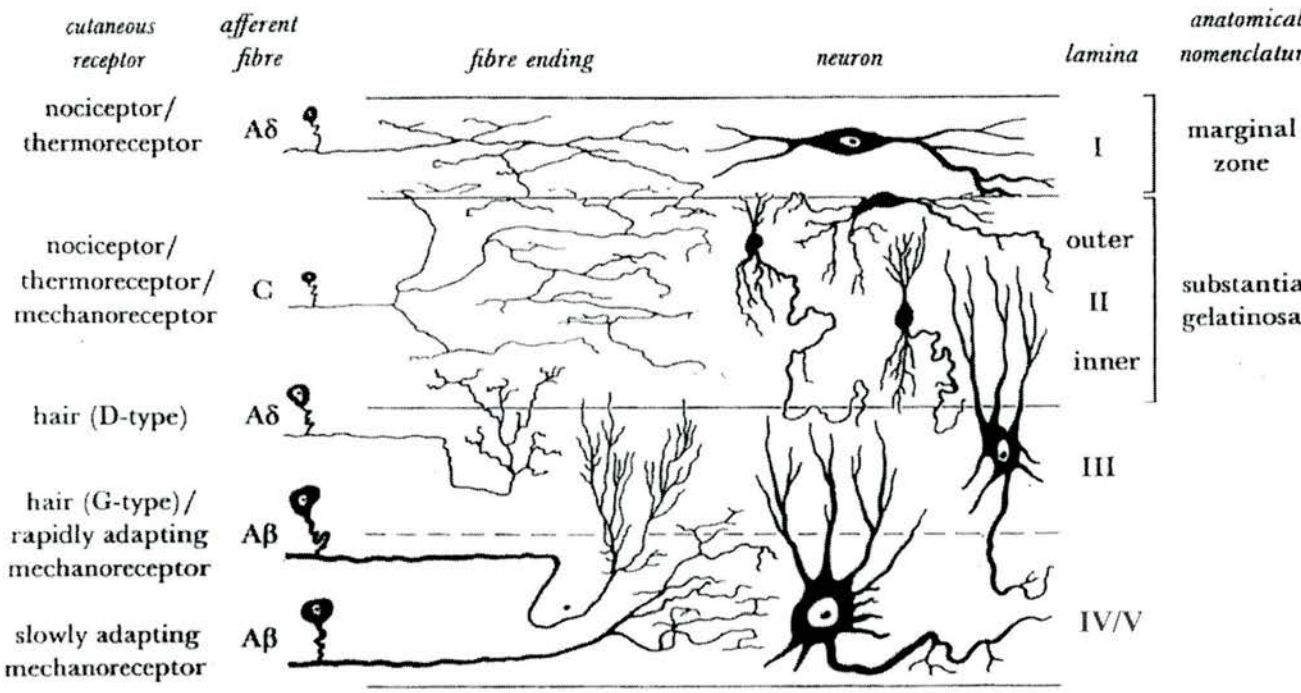
Anatomy of trigeminal sensory nuclei within brainstem



Schematic drawing of the brainstem showing locations of the trigeminal sensory nuclei and some of their connections. Primary afferent fibres are shown to enter brainstem at the level of the pons. Trigeminal ganglion is circled, showing only some of the cells bodies of the primary afferent fibres present within it, other cell bodies located in the mesencephalic nucleus. Trigeminal complex divided into four nuclei principle sensory, oralis, interpolaris and caudalis. Also shown is the trigeminothalamic ascending tract with receives input from all of the nuclei. (Adapted from, Wilkinson, J. L, Neuroanatomy for medical students 2nd ed.Oxford)

Figure 1.3

Schematic diagram of the cutaneous afferent input to, and neuronal organisation of the spinal dorsal horn



A hypothetical cross section of the spinal dorsal horn, illustrating the afferent fibres and neuronal elements present in the first five laminae. The laminar divisions of Rexed (1952) are indicated on the right. Afferent fibre types are listed to the left of the diagram, shown projecting onto neuronal types typical of laminae I-V. The neurones illustrated are (from top to bottom): a marginal cell, an SG limiting cell, two SG central cells and two neurones of the nucleus proprius, the more superficial of which has dendrites penetrating lamina II. (Taken from Cervero and Iggo, 1980)

High-threshold Aδ mechanoreceptors, with thin myelinated fibres, terminate predominantly in lamina I and II of the dorsal horn, as shown here, however they may also have scattered endings which terminate in lamina V of the deeper dorsal horn (Light and Perl, 1979). Similarly, the majority of C-fibre nociceptive afferents terminate in the superficial dorsal horn, particularly lamina II (as shown), a proportion terminate in lamina I and some may project their branches deep down into lamina V (Light and Perl, 1979).

In contrast, non-nociceptive Aδ axons from D-Type hair follicles, and large diameter Aβ myelinated afferent fibres (which innervate sensory cutaneous mechanoreceptors) distribute their axons in the deeper dorsal horn , laminae III-VI (Brown and Iggo, 1967; Perl, 1984), with some endings projecting to the inner portion of lamina II (Brown, 1981; Light and Perl, 1979).

1.6 The Ascending Somatosensory Pathways

Spinal pathways

The Spinothalamic Tract (STT)

This tract was originally thought to be the main pathway for pain transmission due to the analgesia that results from lesions in this area in humans (Willis and Coggeshall, 1978). It projects from cells in laminae I, III, IV, V and VII of the rat spinal dorsal horn to the ventral and posterior nucleus of the thalamus (Giesler et al, 1976; Carstens and Trevino 1978; Willis et al, 1979; Craig and Kniffki, 1985). In the rat, STT cells can respond to noxious and innocuous mechanical stimulation (Giesler et al, 1976), and so the STT is not solely involved in the transmission of nociception and pain. It has recently been shown that in the cat less than 15% of the spinothalamic fibres originate from neurons in lamina I (Klop et al, 2004).

Spinoreticular tract (SRT)

The SRT projects from the spinal dorsal horn to the ventrolateral quadrant of the brainstem reticular formation which in turn relays information to the thalamus (Kevetter and Willis, 1983). HRP mapping has demonstrated that the majority of SRT cells are concentrated in laminae VII and VIII in the rat ventral horn (Chaouch et al, 1983), with some being found superficially. Menetrey et al, (1980) have shown up to 20% of cells in this tract were receptive to noxious stimuli.

Spinomesencephalic Tract (SMT)

The SMT projects to the mesencephalic reticular formation and lateral part of the PAG, as well as to other sites in the midbrain (Mehler et al, 1960). SMT cells are predominantly found in lamina I and V (Menétrey et al, 1982). Electrophysiological evidence has revealed a high proportion of the SMT neurons which originate in the marginal zone, to be nociceptive specific neurons (Menétrey et al, 1980).

Dorsal column pathways

a) Postsynaptic dorsal column system (PSDC)

The PSDC originates primarily from neurons in lamina III of the rat spinal cord (Giesler et al, 1984), and ascend through the dorsal funiculus to the nucleus gracilis and nucleus cuneatus (Giesler et al, 1984). They respond to either innocuous or to both innocuous and noxious stimulation of the cutaneous receptive field (Brown et al, 1983) and also to nocispecific stimuli (Angaut-Petit, 1975).

b) Spinocervical tract (SCT)

SCT cells of origin are mostly located in the deeper laminae (LIV-LVII) and project ipsilaterally to the lateral funiculus. The SCT projects into the PSDC and to deeper regions in the grey matter (Cao et al, 1993). It has been believed that the SCT nucleus in man is absent or vestigial, but post-mortem studies have shown the SCT to exist in 9 out of 16 subjects (Truex et al, 1970). It is concluded that the SCT is a highly variable feature of human anatomy.

It is important to note that these tracts are not entirely separate from each other and that many of these pathways have also been shown to collateralise with each other (Djouhri et al, 1997; Yezierski and Mendez 1991; Cao et al 1993)

Trigeminal pathways

The Trigeminothalamic tract (TTT)

Practically all of the ascending information from the trigeminal nerve goes through this tract, either through its ventral route (pain, temperature and touch) or through its dorsal route (fine touch and position sense). Neurons receiving input from mechanoreceptors, and some nociceptive neurons, project to the ventrobasal nuclei of the contralateral thalamus and then to the sensory cortex. The TTT passes through the medial leminiscus together with ascending pathways from the spinal cord. Cells originate in all of the trigeminal nuclei and all of the zones (Fukushima and Kerr, 1979).

Deep fibre bundle ascending tracts

Evidence from anatomical studies has demonstrated communications of neurons in the SpC projecting to the SpO in the rat in an mainly ascending manner (Esser et al., 1998; Falls, 1984; Jacquin et al., 1990).

More recently, cell bodies of anaesthetised rats labelled retrogradely from the oral subnucleus with the ionophoretically injected tracer tetramethylrhodamine-dextran were observed in laminae III-IV and V of the ipsilateral caudal subnucleus consistently, and to a lesser degree in lamina I. Such a distribution of retrogradely labelled cells suggested that specific subsets of neurons may relay nociceptive information, and others non-nociceptive information. Furthermore, the intra-trigeminal connections conserved the somatotopic distribution of primary afferents in the two subnuclei (Voisin et al, 2002).

Other tracts in the trigeminal system

Other nociceptive fibres make bilateral connections with the reticular formation and non-specific thalamic nuclei. From there they project to many sites in the forebrain.

Some special sensory connections are also present. Taste is relayed by the trigeminal and glossopharyngeal nerves via the trigeminal complex through to the nucleus solitarius (Sessle, 2000; Dubner and Bennett, 1983) and also via the facial nerve (palatal taste receptors) and the vagus nerve (pharyngeal taste receptors) (Scully, 2002).

1.7 Neurotransmitters of Primary Afferent Nociceptors, receptors and their inputs

The excitatory amino acids (EAAs) and several peptides have been implicated in synaptic transmission via primary afferent fibres, and several neuroactive substances have been shown to co-exist within both primary afferents and sensory neurons (Ju et al, 1987; Smith et al, 1993). A large number of agents that mimic putative neurotransmitters have been found to affect the responses of dorsal horn neurons (Willis and Coggeshall, 1991) and trigeminal neurons (Salt and Hill, 1982; Wang et al, 1996; Grudt et al, 1995; Dougherty et al, 1996).

1.7.1 Glutamate and its receptors

The excitatory amino acid glutamate is released in the dorsal horn following noxious stimulation or peripheral inflammation (Skilling et al, 1988; Sorkin et al, 1992) and has been identified immunocytochemically in all somatosensory fibre types (DeBiasi and Rustioni, 1988).

Ionophoretic application of glutamate causes excitation of dorsal horn neurons (Curtis et al, 1959) and evidence has shown that glutamate is released from many types of primary afferent fibres following electrical stimulation or natural stimulation (Roberts, 1974; Ishikawa et al, 2000). In addition, intrathecal administration of glutamate produces behavioural hyperalgesia and spontaneous nociceptive behaviour (Aanonsen and Wilcox, 1986; 1987). This evidence suggests a strong role in the transmission of nociceptive information.

There are several receptor subtypes through which glutamate may mediate cellular effects. They can be grouped as,

a) The ionotropic group of receptor-linked ion channels, consisting of
   (i) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-sensitive receptors
   (ii) N-methyl- D-aspartate (NMDA)-sensitive receptors
   (iii) Kainate acid (KA)-sensitive receptors

b) The metabotropic glutamate receptors (mGluRs), these are coupled through GTP-binding proteins to various second messenger systems.

Ionotropic glutamate receptors

AMPA receptors can be composed of various combinations of four of the GluR 1-4 subunits (Wisden and Seeburg 1993). Their activation leads to a potent depolarisation of dorsal horn neurons. Conversely a blockade of AMPA receptors attenuates synaptic activation of dorsal horn neurons by noxious and non-noxious stimuli (Dougherty et al, 1992), even in normal animals, showing a role in normal somatosensory transmission in the spinal cord. Concentrations of AMPA receptor subunits have been demonstrated in neurons of the superficial laminae of the dorsal horn (Furuyama et al, 1993; Henley et al, 1993; Tölle et al, 1993; Popratiloff et al, 1996) and in the trigeminal nucleus caudalis (Tang et al, 2001; Kondo et al 1995). AMPA receptors are believed to mediate the fast synaptic transmission brought about by glutamate release (Jahr and Jessell, 1985; Jessell et al, 1986; Gerber and Randic, 1989), and in the trigeminal nucleus caudalis the AMPA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) largely attenuates monosynaptic excitatory postsynaptic currents (EPSCs) following electrical stimulation of the mandibular nerve (Onodera et al, 2000).

The NMDA receptor has been shown to play a crucial role in activity-dependant excitability changes such as the homosynaptic facilitation of long term potentiation (LTP) in the hippocampus (Collingridge et al, 1983; Morris et al, 1986) which may be important for "spatial" learning and memory to occur. The mechanism of LTP acting at the single synapse level can be considered a possible parallel but separate mechanism to that occurring in central sensitisation (that is, the facilitation of neuronal activity in response to heterosynaptic input) in the spinal cord (Ji et al, 2003) and the involvement of such mechanisms has been demonstrated in the generation of hyperalgesia (Dougherty et al, 1992; Zhou et al, 1996). The parallels between LTP and spinal sensitisation have, in the past few years, been shown to be more complex with evidence for different forms of plasticity in the spinal cord (Chiang et al, 1998;

Park et al, 2001; Ji et al, 2003; Sandkuhler, 2000). As the NMDA receptor is not normally involved in normal somatosensory processing, it will be discussed in more detail in the section on changes in the central nervous system following nerve injury where it assumes a role.

NMDA application can alter the excitability of spinal neurons (Leem et al, 1996; Haley et al, 1990; Chaplan et al, 1990). In the trigeminal nucleus caudalis, a significant increase in the total c-fibre-evoked activity was observed 15-25 min after the NMDA application for nine neurons located in superficial laminae II and III (Luccarini et al, 2001). In contrast, a significant decrease in the total C-fibre-evoked activity was observed 5-25 min after the NMDA application for 10 neurons located more deeply, in lamina V. This provides evidence for a possible inhibitory role for NMDA-dependent interneurons of the superficial laminae of the trigeminal nucleus caudalis on the nociceptive activity of deep convergent neurons. Also, the injection of NMDA receptor antagonists into the superficial laminae of the trigeminal nucleus caudalis facilitated an electrically stimulated enhancement of c-fibre evoked activity in the trigeminal nucleus oralis recorded simultaneously (Woda et al, 2001) suggesting that these neurons located in substantia gelatinosa of trigeminal nucleus caudalis, exert, in normal conditions, an inhibitory control on convergent trigeminal nucleus oralis neurons.

KA receptors are composed of GluR5-7 and KA1-2 subunits but their role in somatosensory processing is poorly understood due to the present lack of selective pharmaceutical agents (Lerma et al, 2001). As well as in spinal cord (Ruscheweyh and Sandkuhler, 2002) it is known that kainate receptors are expressed in the trigeminal ganglion (Sahara et al, 1997) and that kainate-activated currents in isolated trigeminal complex neurons are inhibited by calcium (Gu and Huang, 1991). It has been reported that activation of kainate receptors on presynaptic terminals reduces glutamate release in the superficial dorsal horn (Kerchner et al, 2001a).

Glutamatergic transmission between intrinsic dorsal horn neurons was also depressed by kainate but not by a selective GluR5 agonist (ATPA). This suggests that intrinsic dorsal horn neurons express another type of kainate receptors than primary afferents that is insensitive to ATPA (Kerchner et al, 2001a). However, some of the inhibitory dorsal horn neurons are sensitive to ATPA (Kerchner et al, 2001b). Indeed, it has been shown that brief activation of postsynaptic kainate receptors on superficial

dorsal horn neurons by stimulation of primary afferents leads to inward currents (Li et al, 1999). Kainate receptors were activated following high-intensity but not low-intensity dorsal root entry zone stimulation, suggesting that kainate receptors are only localized at synapses receiving input from high-threshold primary afferents. Another probably pro-nociceptive mechanism of the activation of spinal dorsal horn kainate receptors involves a reduction of the evoked release of inhibitory transmitters (Kerchner et al, 2001b). The proposed mechanism is rather complex.

Metabotropic glutamate receptors

A number of studies have provided evidence that mGluRs are involved in mediating nociceptive inputs to the spinal cord. Electrophysiological studies revealed that the mGluRs are important mediators in the sustained activation of dorsal horn neurons elicited by topical, cutaneous application of the chemical irritant mustard oil (Young et al, 1994), which produces a selective activation of C-fibres and causes sustained activation of dorsal horn neurons (Lynn, 1997). Further experiments have revealed that the mGluR1 receptor subtype is particularly important in mediating nociceptive transmission at the spinal cord level (Young et al, 1995a; 1995b; 1997; Fundytus et al, 1994) as well as group II mGlu receptors (Fundytus et al, 2002). A selective group I mGluR agonist, in sheep, elicited a significant decrease in threshold values to noxious mechanical stimuli whereas a selective group II mGluR agonist had the opposite effect suggesting contrasting roles in mediating acute nociceptive events in the spinal cord (Dolan and Nolan, 2000). Further, mGluR5 (a group I mGluR member) has been associated with the mediation of aspects of neuropathic pain which may be associated with C-fibre inputs (Dogrul et al, 2000). In the trigeminal system, studies have shown moderate mGluR1 and strong mGluR2/3 immunohistochemical staining in the caudalis lamina II (Tang et al, 2001) and immunoreactivity for mGluR7 (Li et al, 1996) and mGluR1 protein (Araki et al, 1993) in the trigeminal ganglion. In trigeminal neuron sensory roots isolated in brainstem preparations from neonatal rats, mGluR group I agonist increased spontaneous firing, whereas group II and III agonists decreased it (Narita, 1998).

So there are clearly a number of different pathways for glutamate to exert its function as a neurotransmitter within the CNS. Not only several receptor types which lead to different functions of ion transport and intracellular signalling, it can also exert both excitatory and inhibitory effects.

1.7.2 Neuropeptides and their receptors

Substance P (SP) a member of the tachykinin family, is expressed mainly by small diameter neurons in DRG (Hokfelt et al, 1975; Ju et al, 1987). When released, SP activates the G protein coupled NK_1 receptor, dense concentrations of which have been demonstrated within the superficial layers of the spinal cord (Helke et al, 1986; Näsström et al, 1992; Quirion et al, 1983; Yashpal et al, 1991) and in the trigeminal nucleus caudalis (Aicher et al, 2000; Sabino et al, 2002). In addition, at the electron microscope level, NK_1 receptor-immunoreactivity has been found in major dendrites of dorsal horn neurons in laminae III, IV and to some extent V (Brown et al, 1995; Naim et al, 1997) and also in superficial layers of the trigeminal complex (Li et al, 2000). Behavioural studies demonstrate that intrathecal injection of SP in normal mice elicits a dose-related biting and scratching response, which is considered the result of noxious sensations (Hayes and Tyers, 1979; Hylden and Wilcox, 1981; Yashpal et al, 1982). Ionophoretic application of SP increases the excitability of dorsal horn neurons (Henry, 1978; Zieglgansberger and Tulloch, 1979) and trigeminal nucleus caudalis neurons (Salt et al, 1982) and selectively activates high threshold multireceptive lamina I and II neurons in spinal cord (Randic and Miletic, 1977). Substance P abolishes the facilitatory effect of ATP on spontaneous glycine release in neurons of the trigeminal nucleus caudalis (Wang et al, 2001). More recently a reduced aversion to oral capsaicin following neurotoxic destruction of superficial medullary neurons expressing NK_1 receptors has been shown (Simons et al 2002).

However, NK_1 receptor antagonists are generally not effective at reducing dorsal horn neuron responses to brief noxious stimuli (Fleetwood-Walker et al, 1990; Yamamoto and Yaksh, 1991) and have not proved useful as analgesic agents in clinical trials of many chronic pain states (Hill, 2001) Interestingly, micro-dialysis techniques revealed that noxious mechanical and severe thermal stimuli specifically

evoked SP release (Kuraishi et al 1989) and that greater increments in SP levels were recorded following stimuli which caused damage to the peripheral tissues. In addition, antibody microprobe techniques showed a release of SP within the superficial dorsal horn following noxious heat (52°C +), noxious mechanical or chemical stimuli (Duggan et al, 1987; 1988). So the main role for SP and NK_1 receptors in sensory processing therefore appears to be modulators of sustained nociception, due to inflammation.

Calcitonin gene related peptide (CGRP) is yet another constituent of primary afferent fibres, found in approximately 30% of all primary afferent axons (Levine et al, 1993). CGRP-containing fibres are largely unmyelinated (C) fibres or myelinated small diameter (Aδ) fibres, and have been shown to terminate predominantly in laminae I, II and V of the spinal cord (Carlton et al, 1988) and fibres showing moderately dense immunoreactivity can be seen in the human trigeminal nucleus caudalis (Uddman et al, 2002) and in the rat trigeminal ganglion (Ma et al, 2001). CGRP is released in the superficial dorsal horn in response to noxious thermal, mechanical or electrical stimulation (Morton et al. 1990) and is often co-expressed with substance P (Battaglia and Rustioni, 1988; Ju et al, 1987). Concentrations of CGRP, which have little or no consistent effect alone, synergistically increase the effect of SP on rat dorsal horn neurons (Biella et al, 1991), as well as enhancing the release of SP from spinal cord slices (Oku et al, 1987). It is believed that CGRP plays a modulatory role in excitatory transmission in the spinal cord by inhibiting SP endopeptidase, potentiating the effect of co-released SP (Mao et al, 1992). CGRP may also regulate the expression of NK_1 receptors by spinal dorsal horn neurons via gene expression (Seybold et al, 2003).
CGRP is recognised by at least two subtypes of G protein coupled receptors (Wu et al 2002) but can also be recognised by several calcitonin receptors when associated with receptor-associated-modifying-proteins (RAMP) (Born et al, 2002).

Somatostatin (SOM): Somatostatin mRNA positive neuron profiles can be found in the gelatinous subnucleus at the caudal part of the spinal trigeminal nucleus and in the substantia gelatinosa of the dorsal horn (Yin, 1995).

However, electrophysiological studies on spinal neurons *in vivo* and *in vitro* using somatostatin agonists and antagonists have produced a variety of results, demonstrating both an inhibitory (Murase et al, 1982; Randic and Miletic, 1978) and an excitatory (MacDonald and Nowak, 1981b; Salt et al, 1982) role for this peptide within the CNS, so its functional role is not very clear. It is now established that there are at least five types of somatostatin receptor (SSTR1-5), which mediate different actions (Reisine et al, 1995; Carlton et al, 2001; Song et al, 2002; Jiang et al, 2003). It has been suggested that SOM may affect the release of other transmitters (MacDonald and Nowak, 1981a), which may partly explain the discrepancies in the electrophysiological studies and would suggest that the primary role for this peptide may be as a modulator rather than a transmitter, or again that it has different actions on different SOM receptor types. Recently, in situ hybridisation has demonstrated SOM expression to be localised in lumbar DRG neurons of the rat, all of which are intensely IB4-labeled neurons and express the GNDF receptor (Kashiba et al, 2001). Application of GDNF to the rat dorsal horn of the spinal cord promotes activity-induced release of SOM from central terminals of sensory neurons (Lever and Malcangio, 2002) and GDNF-induced inhibition of intrathecal SP-induced thermal hyperalgesia was reversed by the SOM antagonist (Lever and Malcangio, 2002).

Vasoactive intestinal polypeptide (VIP): Under normal conditions, VIP expression is seen only at very low levels in DRG neurons and in LI and LII neurons of the spinal dorsal horn of the rat (Gibson et al, 1981;Knyihar-Csillik et al, 1991) and is absent in the human trigeminal nucleus caudalis (Uddman et al, 2002). There is a clear rostro-caudal gradient of the autoradiographic binding sites for VIP in spinal cord, with the greatest concentrations generally found at lower lumbar to sacral levels (Gibson et al, 1981; Yashpal et al, 1991b). It activates the receptors $VPAC_1$, $VPAC_2$ and to a lesser extent PAC_1 (Hosoya et al, 1997; Ishihara et al, 1992). When applied to single neurons ionophoretically, VIP and PACAP produce an excitatory effect (Salt and Hill, 1981; Dickinson et al, 1999). Intrathecal application of VIP has been shown to facilitate nociceptive input at the spinal cord level, as studied on flexor reflexes (Cridland and Henry, 1988; Wiesenfeld-Hallin, 1987).

Neuropeptide Y (NPY): There is a high density of NPY-positive nerve fibres present in the superficial laminae of the dorsal horn arising from local cell populations or supraspinal sites (Wakisaka et al, 1991), where NPY may co-exist with galanin, GABA (Laing et al, 1994; Zhang et al, 1993), or 5-HT (Van Dongen et al, 1985). The functional role of NPY in the spinal cord is unclear, as at low doses intrathecal administration of NPY appears to be excitatory, whereas at high doses, it produces inhibitory actions (Ullstrom et al, 1999). This is also complicated by the discovery of six different receptors to date, Y1-5 and Y6, which is not functional in humans (Silva et al, 2002). Microinjection of NPY in the dorsal horn has been shown to evoke a stimulus-induced decrease in SP release from primary afferents (Duggan et al, 1991), suggesting that NPY could cause pre-synaptic inhibition of primary afferent transmitter release. In addition, NPY administered intrathecally can produce analgesia in conscious rats (Holets et al, 1988). The majority of NPY-containing neurons within the dorsal horn have been shown to be GABAergic (Rowan et al, 1993), and NPY may therefore be acting in conjunction with GABA in order to produce pre-synaptic inhibition.

Cholecystokinin (CCK): Cholecystokinin is expressed in small diameter neurons in the DRG, as well as in peripheral nerves and the superficial layers of the dorsal horn (Fuji et al, 1983; 1985). It is expressed in relatively low levels in the normal animal (Noguchi et al, 1993). In the trigeminal nuclei, immunoreactive CCK fibres were present in all three subnuclei, but the greatest volume fraction of immunoreactive axons was obtained in laminae I and II of the trigeminal nucleus caudalis. Co localization of cholecystokinin and substance P was also shown (Clements et al, 1987). No immunoreactive cell bodies were evident in any of the subnuclei suggesting the possibility that the CCK-immunoreactivity present might come mostly from primary afferents, a view substantiated by findings of relatively low levels of CCK-immunoreactivity in other brainstem nuclei with connections to the trigeminal complex (Beitz et al, 1987).

Galanin: Galanin is a 29 amino-acid peptide that is normally expressed in small to medium diameter DRG neurons, where it co-exists with several other neuropeptide transmitters including CGRP and SP (Ju et al, 1987; Zhang et al, 1993; 1995). While galanin-containing neurons and nerve fibres occurred frequently in the trigeminal ganglion and trigeminal nucleus caudalis in human newborns, they were scarce and/or

absent in adults (Del Fiacco and Quartu, 1994). Rapid and marked increases of preprogalanin mRNA was also seen in the trigeminal nucleus caudalis following formalin injection (Tokunaga et al, 1992).

Galanin levels have been shown to decrease in the superficial laminae (LI-II) of the dorsal horn following neonatal cutaneous capsaicin treatment (Skofitsch and Jacobowitz, 1985), suggesting that spinal galanin arises substantially from primary afferent C fibres. Galanin may act centrally as a modulator of excitatory peptides, supported by evidence that intrathecal pre-administration of galanin antagonises the excitatory effects of SP and CGRP on the flexor withdrawal reflex (Wiesenfeld-Hallin et al, 1991b; Xu et al, 1990) and inhibits the analgesic effect of morphine on noxious thermal and mechanical stimuli (Wiesenfeld-Hallin et al, 1991a), whilst producing no effect alone on nociceptive inputs. It is thought that galanin is important during development and recent work has shown that mice lacking the gene for galanin have 15% fewer DRG neurons than wild-types and that these neurons regenerate more slowly after injury and have fewer branches when cultured (Wynick et al, 2001). Three receptors for galanin have been discovered GAL1-3 (Branchek et al, 2000),of which, only GAL1 and 2 seem to be expressed in the neuronal membrane (Parker et al, 1995; O'Donnell et al, 1999). GAL1 receptors are inhibitory and are present both pre and post-synaptically. GAL2 receptors are excitatory, pre-synaptic receptors. To date there have been no specific studies of the action of galanin in the trigeminal complex.

In rats intrathecal galanin produces brief facilitatory effects at low doses, which become inhibitory as the dose increases, such that at very high doses galanin exerts a purely inhibitory effect (Weisenfeld-Hallin et al, 1988; 89). In addition, in both electrophysiological and behavioural studies, galanin was seen to inhibit the analgesic effect of morphine on noxious thermal and mechanical stimuli (Weisenfeld-Hallin et al, 1990c), while having no effect alone on these nociceptive inputs. Galanin may also be an important modulator of excitatory neuropeptide action, as administration of galanin intrathecally has been reported to antagonise the excitatory effects of SP and CGRP on the flexor reflex (Weisenfeld-Hallin et al, 1990a; Xu et al, 1989; 1990). In addition, the VIP-induced flexor reflex could be inhibited by galanin antagonists following axotomy (Xu et al, 1990). These results might be explained as actions of different receptors.

1.8 Descending and local control of nociceptive transmission in the spinal dorsal horn and trigeminal complex.

1.8.1 Descending inhibition

There are several regions in the central nervous system that exert an inhibitory control over nociceptive input. It has been shown that morphine micro-injection into, and electrical stimulation of, the periaqueductal gray area (PAG) inhibits the responses of wide dynamic range and nociceptive specific neurons in L1-II and VII (Mayer and Price 1976, Sessle et al, 1981). Anatomical studies (Gallagher and Pert 1978, Abols and Basbaum, 1981) have shown direct projections from the PAG to the nucleus raphe magnus (NRM), and the reticular nuclei (RN). Projections from the NRM and the RN to the medullary and spinal dorsal horns have been described (Basbaum et al, 1978). The NRM provides a major serotonergic input to the dorsal horn LI/II and V cells (Basbaum and Fields, 1978), electrical stimulation of which can produce analgesia to noxious stimuli (Guilbaud et al, 1977; Willis, 1977; Oliveras et al, 1974; Duggan and Griersmith, 1979), and it has been shown that 5HT1A (Deseure et al, 2002) and 5HT1B/1D (Kayser et al, 2002) receptor agonists can attenuate pain related behaviours in a trigeminal neuropathic pain model. But NRM lesions have been shown to have no effect on behavioural analgesia induced by morphine or electrical stimulation in the PAG (Hall et al, 1981) unless the lesion extended into the magnocellular reticular field (Cannon et al, 1980), suggesting the NRM participates, but is not essential in the mediation of descending effects.

Electrical stimulation in the rostro-ventromedial medulla (RVM) has also been shown to influence descending control of nociceptive transmission and produces a biphasic modulatory effect, showing facilitation at low intensities and inhibition at higher intensities (Calejesan et al, 2000). Fields and Heinricher (Fields and Heinricher, 1985) demonstrated two main classes of cell in the RVM namely; 'on-cells', which are constantly activated by noxious heat stimulation of almost anywhere on the body surface and 'off-cells', which are inhibited by the same stimulus (Hernandez and Vanegas, 2001). A similar division of cell type has been demonstrated in the parafascicular neurons (PF) of the thalamus (Zhang et al, 1997), which is modulated by ascending and descending influences of the locus coeruleus (LC), that provide

noradrenergic input to the forebrain, cerebellum, brainstem, spinal cord (Nygren, 1977), and trigeminal complex (Senba et al, 1981). The LC exerts two different effects on nociceptive transmission via the PF; a predominantly inhibitory role on nociceptive transmission at the level of the spinal cord by descending noradrenergic fibres, and a facilitatory role on the responsiveness of PF to noxious inputs by ascending fibres (Zhang et al, 1997). Studies in the trigeminal complex indicate a noradrenalin-mediated inhibitory influence of the LC on neurons in the trigeminal nuclei (Sasa et al, 1974 and 1979) It has been proposed that these regional interactions may be responsible for the phenomena of diffuse noxious inhibitory control (DNIC) where a variety of noxious stimuli applied to remote body sites inhibit the activity of wide dynamic range trigeminal and spinal dorsal horn neurons (LeBars et al, 1979, 1981; Dickenson et al, 1980). DNIC results from a physiological activation of some of the brainstem structures that produce descending inhibition. Surprisingly, DNIC were not modified by lesions of the periaqueductal grey (PAG), cuneiform nucleus, parabrachial area, locus coeruleus/subcoeruleus or rostroventral medulla (RVM). By contrast, lesions of subnucleus reticularis dorsalis (SRD) in the caudal medulla strongly reduced DNIC (Le Bars et al,1992; Villanueva and Le Bars, 1995). The SRD is located ventral to the cuneate nucleus, between trigeminal nucleus caudalis and the nucleus of the solitary tract and contains neurones with characteristics which suggest that they have a key role specifically in the processing of nociceptive information (Villanueva et al, 1996). Therefore, descending modulation of nociceptive transmission in the spinal cord involves complex integration between various brain structures.

1.8.2 Segmental control

Segmental controls have been observed as the inhibitory effects produced by large diameter (Aβ) fibres on the responses of spinal neurons to nociceptive stimulation (Brown et al 1973; Cervero et al, 1976) and these inhibitory influences can be exerted on multireceptive and nocispecific dorsal horn neurons. Inhibitory transmitters present in the spinal cord and brainstem include the inhibitory amino acids γ-aminobutyric acid (GABA), glycine and the endogenous opioids. These neurotransmitters may be released by interneurons following activation from not only the descending pathways but also from primary afferent fibres (Curtis et al, 1977: Gmelin and Cerletti, 1976). Furthermore, monoamines such as, serotonin and noradrenalin, may be directly

released by descending fibres to exert inhibitory effects (Basbaum and Fields 1978; 1984).

GABA is found in approximately one-third of laminae I, II and III dorsal horn interneurons (Barber et al, 1982; Hunt et al, 1981; Todd and McKenzie, 1989), as well as in neurons of the rostral ventro-lateral medulla which project to the spinal cord (Reichling and Basbaum, 1990). Ionophoresis of GABA results in inhibition of dorsal horn neuron activity, including those in the substantia gelatinosa (Curtis et al 1959; 1977; Zieglgansberger and Sutor, 1983).

In the trigeminal nucleus caudalis, GABA is present in a subpopulation of presynaptic axonal terminals within lamina II where it co-localizes with glycine (Dumba et al, 1998). In the trigeminal nucleus caudalis, GABA (A) receptor blockade by intrathecal bicuculline in rats treated with mustard oil, produced a significant increase in spontaneous activity and in pinch and tactile receptive field (RF) size (or the appearance of a tactile area in the RF of nociceptive-specific neurons), as well as a significant lowering of the mechanical threshold and a significant enhancement of responses to pinch stimuli applied to the RF (Chiang et al, 1998). It is also thought that interneurons in LII in the spinal dorsal horn, many of which are inhibitory, may die after partial nerve damage, further increasing disinhibition (McLachlan et al, 1993; Sugimoto et al, 1990; Willis and Coggeshall, 1991; Moore et al, 2002). However some evidence suggests that this is not necessary for the development of thermal hyperalgesia (Polgar et al, 2003).

Glycine expression is found in LI-III (predominantly LIII) of the spinal cord (Todd and Sullivan, 1990) and the trigeminal nucleus caudalis (Dumba et al, 1998). As well as acting via the strychnine-sensitive inhibitory glycine receptor (Budai et al, 1992), glycine can also exert an excitatory action as a cofactor for the *N*-methyl-D-aspartate (NMDA) receptor. However, ionophoretic application of glycine results in strong depression of dorsal horn neuron activity (Zieglgansberger and Sutor, 1983).

GABAergic and glycinergic neurons have been examined with both the light- and electron-microscope in the trigeminal nucleus caudalis in the rat. The majority of GABA- and glycine-immunoreactive neurons showed co-localisation in both spinal cord (Todd and Sullivan, 1990), and the trigeminal complex (Wang et al, 2000).

Endogenous opioid peptides such as enkephalin, dynorphin and nociceptin are present within synaptic terminals of the spinal dorsal horn (Cruz and Basbaum, 1985; Glazer and Basbaum, 1981; Willis and Coggeshall, 1991). Opioid receptors of μ and δ subtypes have been localised in the superficial dorsal horn and κ receptors in deeper regions of the spinal cord (Atweh and Kuhar, 1977; Calza et al, 2000; Dun et al, 2000; Aicher et al, 2000; Zhang et al, 2000). Opioids can act directly on the spinal cord, and ionophoretic injection of opioids inhibits spinal nociceptive processing (Duggan and North, 1983; Fleetwood-Walker et al, 1988). This inhibitory action may be pre-synaptic or may be in response to activation of opioid-containing interneurons. However, opioid drugs appear to be poorly effective in neuropathic pain states (Arner and Meyerson, 1988), possibly because following nerve damage, opioid receptors are seen to be down-regulated (Woolf and Mannion, 1999).

It has been shown that intrathecal morphine inhibits substance P release from mammalian spinal cord in vivo, (Yaksh et al, 1980), and that opiate analgesics inhibit substance P release from rat trigeminal nucleus (Jessel and Inversen, 1977). Similarly morphine alone, has been shown to be enough to reduce C-fibre evoked activity in the trigeminal oralis (Dallel et al, 1996; Duale et al, 2001) in halothane anaesthetised rats. Early reports showed that intra-venous morphine produced both a reduction in nociceptive activity of some neurons in the trigeminal nucleus caudalis but also excitation in others (Ayliffe and Hill, 1979), and more recently, micro-injection of opioids into the SpO of the trigeminal complex has been shown not to inhibit C-fibre evoked responses of SpO neurons but that injection into the SpC does inhibit SpO neuron sensitisation (Dallel et al, 1998). Furthermore, others have shown, that morphine micro-injection into the SpO may exert an anti-nociceptive effect on the formalin test (Luccarini et al, 1995).

There has not been a systematic review of the distribution of opioid receptors in the trigeminal literature. But experiments have been carried out on co-localisation studies with mu-opioid and NMDA receptors and distribution of single receptors types like the delta-opioid receptor (Aicher et al, 2002; Kalyuzhny et al, 2000). Correspondingly, Christensen et al, (1999) have shown, in a rat model of neuropathic pain, that combined administration of both an NMDA receptor glycine antagonist and

morphine synergistically attenuated allodynia. Mu-opioid receptors in the trigeminal system have also been shown to be in SP and CGRP-ir fibres (Li et al, 1998) and to co-localise with NMDA receptors (Aicher et al, 2002). The delta-opioid receptor has also been shown to be diffusely distributed throughout the trigeminal system (Arvidsson et al, 1995a) and it has been concluded that mu- and delta-opioid receptor agonists produce a predominantly inhibitory modulation of the NMDA-evoked responses of nociceptive and non-nociceptive neurons in the medullary dorsal horn (Zhang et al, 1996).

A complex interaction of opioids with NMDA receptors may occur, however, with mu-opioid receptors shown to modulate NMDA activity through PKC (Chen and Huang, 1991), and endogenous opioid ligands shown to actually block the NMDA receptor at higher concentrations (Chen et al, 1995) by interacting with a site conformationally linked with the redox site(s), thus altering the gating properties of the channel.

In contrast to earlier reports of morphine administration inhibiting SP release from spinal cord (Jessell and Inversen, 1977), it has been reported that activation of kappa opioid receptors by U50488H and morphine enhances the release of substance P from rat trigeminal nucleus slices (Suarez-Roca and Maixner, 1993). This could be due to differential distribution of individual opioid receptors in the dorsal horn (Fleetwood-Walker et al., 1988; Gouarderes et al., 1996; Kalso et al., 1993;Sullivan et al., 1994; Kieffer and Gaveriaux-Ruff, 2002). Recently it has been demonstrated that some patients with trigeminal neuropathic pain responded well to intravenous injection of a kappa-opioid agonist and naloxone when they previously had no benefit from mu-opioid agonists (Schmidt et al, 2003). This apparent paradox may still be rationalised because following neuropathy SP is known to decrease ipsilateral to nerve injury which suggests that a switch away from the influence of SP-containing neurons occurs following nerve injury (Nahin et al, 1994; Hokfelt et al, 1994). Futhermore, an increase in preprodynorphin mRNA was observed on the side ipsilateral to the injection, but a less pronounced change in preproenkephalin-A mRNA level was evident, indicating that these peptides have different roles in analgesic mechanisms (Tokunga et al, 1992).

In normal circumstances these mechanisms for processing nociceptive inputs in spinal and medullary dorsal horn alert the organism to actual and potential damage and acute

pain serves to protect the organism from this damage. However, following nerve injury other changes take place which result in a persistent pain that outlasts the stimulus even beyond the time taken for healing of the tissues. This, neuropathic pain, does not serve to protect the organism and are potentially debilitating.

1.9 Neuropathic pain

Neuropathic pain is pain felt as a consequence of nerve damage. Nerve damage can cause normal sensory transduction and processing to be altered. These alterations may be responsible for symptoms such as spontaneous pain (stimulus-independent pain) and hypersensitivity to a stimulus (stimulus-evoked pain). This hypersensitivity includes allodynia (the perception of normally innocuous stimuli such as touching or brushing as painful) and hyperalgesia (an increased pain response to a normally noxious stimulus).

Several theories of pathophysiology have been proposed to explain trigeminal neuralgia's (TN) clinical features and the efficacy of vascular decompression surgery. These theories, which focus on alterations either in the trigeminal ganglion or more centrally, include epileptogenic activity, reverberating circuits, ephaptic connections, and changes in central connectivity (Loeser, 1989). The most recent and detailed theory was proposed by Rappaport and Devor (1994) to account for almost all clinical features of TN. They hypothesized that compression-induced damage to the trigeminal root leads to hyperexcitability of a small cluster of trigeminal ganglion neurons, which, in turn, forms an 'ignition focus' that spreads to more regions of the ganglion. What is unanswered, however, is how any compression-induced excitability would enable activity in low-threshold sensory fibres to induce paroxysmal pain that would normally only be evoked by a massive barrage of activity in high threshold C-fibre nociceptors. This paradox lies at the heart of the pathophysiology of TN: how does a normally innocuous input activate severe paroxysmal pain? There is certainly good evidence that the tactile allodynia (brush-evoked pain) in other neuropathic pain is mediated by low-threshold mechanoreceptors (A-beta fibres) (Woolf and Mannion

1999), but there is no comparable situation where light touch provokes paroxysmal pain.

Vascular compression of the central axons of the trigeminal nerve, typically at the root entry zone near the pons, has been inferred to be a possible cause of TN in most patients. The resulting demyelination is postulated to alter the electrical activity of trigeminal neurons. Vascular compression combined with signs of demyelination or nerve injury are found in the overwhelming majority of surgical patients (Jannetta, 1967, Burchiel, 1980; and Hamlyn and King, 1992). When the vessels (mostly arteries, but occasionally veins) are separated from the nerve and/or removed through microvascular decompression, patients' paroxysmal pain disappears almost immediately (Barker et al, 1996). Magnetic resonance imaging studies, which have only in the last few years permitted preoperative visualization of neurovascular relationships, reveal vascular contact with the nerve in a high proportion of surgical patients (Meaney et al 1995).

1.10 Animal models of Neuropathic pain.

Several animal models that display features of human neuropathic pain exist. The chronic constriction injury model (CCI) is one of the most commonly used and involves the tying of loose chromic gut ligatures around the full width of the sciatic nerve (Bennett and Xie, 1988) or the infraorbital nerve (Vos et al, 1994). The ligatures only just constrict the nerve and this maintains the blood supply, therefore, many of the axons are left in continuity. Animals show neuropathic pain behaviours within one week following surgery, with the hind paw or side of the face being hyperalgesic and allodynic. The animal also guards the affected area in a protective fashion, a behaviour thought to be associated with the presence of spontaneous pain. Changes become pronounced from 3-4 days following surgery in the sciatic nerve model (and from 9-14 days in the trigeminal model) and can last up to 3 months (and up to 3 months longer in the trigeminal model) the reasons for this difference remain unclear. In the spinal cord there is probably some involvement of the sympathetic nervous system (SNS) as surgical sympathectomy alleviates thermal responses, but so

far this has not been shown to alter mechanical responses (Desmeules et al, 1995, Attal et al, 1990).

Other models of neuropathic pain include; Partial nerve ligation (PNL), after the tight ligation of part of the sciatic nerve (Seltzer et al, 1990), animals display abnormal grooming of the affected hind limb, including licking and biting, as well as holding the affected hind limb in a protective manner, which is thought to be indicative of the presence of spontaneous pain. No signs of autotomy are apparent in PNL animals. It also appears that the SNS contributes significantly to the development of these pain related behaviours, as chemical or surgical sympathectomy has been shown to alleviate or prevent the development of neuropathic pain (Shir and Seltzer, 1991). Animals having undergone PNL show no signs of cold allodynia but do show hyperalgesia and mechanical allodynia. These animals also develop sensitivity bilaterally. Sympathectomy in this model prevents the development of chronic neuropathic sensitisation.

Spinal nerve ligation (SNL), which involves the tight ligation of spinal nerves contributing to the common sciatic nerve just distal to the DRG (Kim and Chung, 1992), also produces abnormal grooming and guarding behaviours with a contribution of the SNS and an absence of autotomy (Na et al, 1996; Choi et al, 1994). In the SNL model, pain onset is rapid (1-2 days) and can last up to 5 months. However, no cold allodynia is present and sympathectomy almost completely abolishes the pain behaviours (Choi et al, 1994).

More recently a variant of partial denervation has been developed called the spared nerve injury model (Decosterd and Woolf, 2000), involving a lesion of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact. Therefore, in this model the co-mingling of distal intact axons with degenerating axons is restricted, permitting behavioural testing of the non-injured skin territories adjacent to the denervated areas.

All the above models produce slightly different expressions of behaviour. The CCI model was used in the present study as it is the model which represented most prominent signs of ongoing pain (Kim et al, 1997), has least involvement from the

SNS, and has been the best described in both spinal and trigeminal nerves. The CCI model is also more aligned to many clinical conditions as it emulates damage to an entire branch of a peripheral nerve which is what is commonly seen after limb damage or damage following fractures to the facial bones. It has also been postulated to be the closest model to the prevailing theory of trigeminal neuralgia aetiology (i.e. compression of the root of the trigeminal nerve by loops of the superior cervical artery) and to give comparable levels of demyelination of the peripheral nerve to that seen in post mortem studies of the trigeminal root of trigeminal neuralgia suffers. Indeed, key features of trigeminal neuralgia have been modelled by constrictive injury of the infraorbital nerve (Vos et al 1994; Benoist et al, 1999).

Aetiology of nerve injury in the CCI model.

Several aspects of nerve injury may contribute to the development of the pain states in these models.

-The variety of material used for ligatures may be important (Kajander et al, 1996). Between chromic gut, plain gut and polygalactin sutures in the CCI model, it was found that the use of chromic gut sutures resulted in a decrease in the levels of CGRP- and SP-immunoradioactivity in the spinal cord, while plain gut and polygalactin sutures did not (Xu et al, 1996). As all types of suture produce abnormal pain behaviours, the physical constriction is likely to be the most important factor, but, it is also apparent that some constituent in chromic gut (most likely, chromic acid) has an additional influence. Changes in local blood flow throughout the injured nerve may also contribute to sensory changes, as there is some evidence of decreased blood flow at the nerve injury site during the time thermal hyperalgesia is evident (Myers et al., 1993). It is suggested that if an acidic environment around the nerve is provided for several days (and an associated inflammatory response ensues), progressive thermal hyperalgesia will develop (Maves et al, 1995).

This inflammatory response appears to be important in the development of neuropathic pain states, as daily injections of the anti-inflammatory steroid dexamethasone, decreased not only the inflammatory response but also guarding behaviour and thermal hyperalgesia in rats (Clatworthy et al, 1995).

1.10.1 Changes in the Peripheral Nervous System

Ectopic activity and altered ion channel expression in peripheral nerves

As a result of nerve injury, some primary sensory neurons generate spontaneous and aberrant action potentials in abnormal regions of the nerve (ectopic activity).

Both myelinated A-fibres and unmyelinated C- fibres can show an emergence of ectopic activity following nerve damage (Devor and Seltzer, 1999; Boucher et al, 2000; Liu et al, 2000), however, it is also clear that intact neighbouring nerve fibres can exhibit spontaneous ectopic activity (Boucher et al, 2000; Michaelis et al, 2000). This activity can occur at the site of nerve injury (Tal and Devor, 1992) and in the cell bodies (Wall and Devor, 1983).

The action potential generated within a nerve membrane occurs as a result of inward and outward currents of Na^+, K^+, Cl^-, and Ca^{2+} ions. Probably the two most important of these channels are the Na^+ and K^+ channels as they are both very widely expressed and are responsible for most of the action potential profile generated in many cells.

In the normal state, DRG neurons express a complex repertoire of Na^+ channel transcripts (Waxman et al., 1999b), which are distinguished by their sensitivity to tetrodotoxin (TTX) (Black and Waxman, 1996). Some of these transcripts, such as TTX-resistant Na^+ channels SNS 1/Na_v1.8 (Akopian et al., 1996) and SNS 2/Na_v1.9 (Tate et al., 1998) are known to be specifically expressed by sensory neurons and are therefore implicated in pain states (Akopian et al., 1996;Novakovic et al., 1998). SNS 1/Na_v1.8, which is of particular interest to this study, has been shown to be expressed in approximately 50% of small diameter unmyelinated (Amaya et al., 2000) and in approximately 20% of medium to large diameter myelinated cells of the DRG, whereas SNS 2/Na_v1.9 is expressed only in neurons with unmyelinated axons (Amaya et al., 2000). Another Na^+ channel of interest to this study is the brain type III Na^+ channel/Na_v1.3, which is normally only found in DRG during development. However, following axotomy (Waxman et al., 1994b) and CCI (Dib-Hajj et al., 1999), changes are seen in the expression of such Na^+ channels resulting in changes in the properties of the Na^+ current. Specifically, the expression of type III/Na_v1.3 is up-regulated in sensory neurons (Black et al., 1999), whereas the two known TTX-resistant channels in DRG neurons, SNS 1/Na_v1.8 and SNS 2/Na_v1.9 are down-regulated (Decosterd et al., 2002;Waxman et al., 1999a).

The SNS class of Na^+ channel produces slowly inactivating currents (Akopian et al., 1996;Waxman et al., 2000), therefore their decreased expression in DRG neurons may lead to a shift in resting potential, (Dib-Hajj et al., 1999;Waxman et al., 2000). Up-regulation of the type III/$Na_v1.3$ Na^+ channel results in a switch in the properties of the TTX-sensitive currents in DRG neurons, with the emergence of a rapidly repriming current (Waxman et al., 1999b), which could sustain frequent ectopic discharges (Cummins and Waxman, 1997). Therefore, DRG neurons that express type III/Na_v 1.3 should be able to sustain higher firing frequencies, which may lead to hyperexcitabilty in the cell.

The Shaker-related potassium channels; $K_v1.1$, $K_v1.2$ and their associated β2 subunit, are concentrated in the region on the internodal side of the paranode; the juxtaparanode (Rasband et al, 1998; Vabnick and Shrager, 1998). These channels have been shown to contribute to rapid axonal membrane repolarisation during development (Vabnick et al, 1999) and are thought to have an important physiological function in dampening the excitability of myelinated fibres, preventing repetitive ectopic discharging.

The expression of ectopic activity differs between sciatic nerve and trigeminal preparations, and indeed, even between different trigeminal nerve preparations. In the rat, ectopic activity of myelinated fibres in sectioned sciatic nerves showed much higher levels than in sectioned infraorbital nerves (maxillary branch of trigeminal nerve) (Tal and Devor, 1992) and the level of activity in the infraorbital nerve did not have a clear relationship with the post-injury period as it did in the sciatic nerve. Several different types of injury to the inferior alveolar nerve (IAN); (a mandibular branch of the trigeminal nerve), in the ferret, give rise to an earlier peak of spontaneous activity with a more accelerated decline compared to sciatic nerve injuries (Bongenhielm and Robinson, 1996 and 1998). Again in the ferret, the lingual nerve (also a part of the mandibular branch of the trigeminal nerve) shows yet another pattern of ectopic activity with a significantly higher level of abnormal activity in the later stages (3 months) post-surgery than in the inferior alveolar nerve (Yates et al, 2000).

It has been postulated that these differences may be due to either the relatively short distance from the injury site to the trigeminal ganglion, the difference in embryonic origin of the DRG and trigeminal ganglion, or a difference in fibre types present (Tal

and Devor, 1992). Also, others have suggested that differences in the inferior alveolar nerve may be related to the IAN itself and its unusual anatomy. As described earlier in the introduction, it is a branch of a cranial nerve carrying sensory afferent fibres and a few sympathetic efferents and it lies within a bony canal. It is markedly different from a limb nerve, such as the sciatic, which is a mixed nerve laying within soft tissues. Some authors suggest that the support of the bony canal around the IAN prevents retraction after injury, maintains apposition of the nerve stumps and increases the potential for regeneration (Elcock et al, 2001a) however, this does not address the fact that the bony canal may disrupted during the experimental protocol.

Ephaptic crosstalk in peripheral nerves following injury

If nerve damage is severe enough, Schwann cells on myelinated fibres may degenerate (Gautron et al, 1990). This can compromise the normal fibre insulation and if so, electrical exchange between nerve fibres may take place (ephaptic crosstalk) (Rasminsky, 1978). As neighbouring fibres are frequently of different types, the possibility exists that small A-δ fibres normally activated in response to a noxious stimulus may suddenly be stimulated by large A-β fibres conveying low threshold mechanical stimuli. This may mean that pathways normally only associated with noxious stimuli can become active due to non-noxious stimuli such as touch at the site of injury in the periphery (Fried et al, 1993; Seltzer and Devor, 1979).

Sympathetic nervous system involvement

Some forms of chronic pain are dependent on the activity of the sympathetic nervous system. Termed Sympathetically Maintained Pain (SMP) it is usually treatable via pharmacological or surgical intervention. Yet, the role of the sympathetic nervous system in the generation and maintenance of chronic pain states in general is still not understood despite considerable work (Janig 1995). It is known that following sciatic nerve transection there is sprouting of sympathetic nerve fibres in the DRG. These new fibres are catecholaminergic axons, and surround the DRG cell somata tending to prefer large cells that have been lesioned and are rare around small cells (McLachlan 1993; Janig, 1996). It has been postulated that activity in these sympathetic neurons could lead to an increase in activity in cells of the DRG. However, in the trigeminal

system no change in the sympathetic fibres after nerve injury has been found, and therefore no sprouting into the ganglion analogous to that in the DRG is seen (Bongenhielm et al, 1999; Benoliel et al, 2001). Even though it can not be ruled out with the evidence to date that there is not involvement of sympathetic neurons that are not directly associated with the ganglion, this may still have profound implications for causalgia (a severe form of regional sympathetic dystrophy or sympathetically maintained pain) which occurs less frequently in the trigeminal region than the limbs (Hoffman and Matthews, 1990). CCI has been shown to increase local blood flow around the site of injury to the nerve via nitric oxide release (Levy and Zochodone, 1998) and also to increase skin blood flow to the ipsilateral paw (Daemen et al, 1998). This increase in local blood flow is part of a local inflammatory reaction which has been postulated to lead to at least some of the sensitisation of peripheral afferent nociceptors.

Nerve cell sprouting.

Following peripheral nerve damage, Woolf et al. (1992) reported that sprouting of A-β fibres or interneurons may occur from deeper laminae into lamina I and the dorsal part of lamina II, and it has been suggested that this could contribute to allodynia associated with neuropathic pain. It is argued that this information from tactile fibres associated with touch may be misinterpreted as nociceptive by the lamina II neurons and this may provide the basis for mechanical allodynia (Woolf and Mannion, 1999). Part of the evidence for sprouting was on the basis of the use of cholera toxin B subunit as a selective tracer for A-fibres, and the validity of this approach has recently been questioned and it has been proposed that this result may in fact be an artefact of the tracer technique used (Tong et al, 1999).

Recently it has been shown, with intra-axonal labelling, that both ipsilateral and contralateral to a sectioned nerve, arbors of axons with hair follicle afferent-like morphology in the sciatic territory extended only as far as the ventral half of lamina II (Hughes et al 2003), and that fine unmyelinated afferents may well be able to transport cholera toxin B after injury (Shehab et al, 2003). Therefore these results do not support the hypothesis that A-β fibre afferents sprout into the superficial laminae after nerve section.

Two main stages of change occur in the nerve after chronic constriction injury (Coggeshall et al, 1993). The first is an early degenerative stage, which is thought to be a direct result of inflammation following ligature placement and as the swelling component increases there is a slow strangulation of the nerve by the ligatures. This can last up to 28 days and by this time a neuroma is often apparent (Coggeshall et al, 1993). The sciatic nerve undergoes marked anatomical changes, distal to the site of nerve injury, at this time. From day 3 to two weeks after nerve ligation, there is a steady and extensive decrease in axon numbers of all types. The most prominent change is a profound loss of large myelinated axons, namely the Aβ fibres, distal to the lesion while the smaller myelinated and unmyelinated fibres appear to be less affected. At the electron microscope level, the few remaining large diameter Aβ fibres were seen to be in an advanced state of degeneration (Basbaum et al. 1991). It is postulated that this loss of large myelinated afferents may lead to at least some loss of inhibitory central input. The second stage is a regenerative phase, and occurs from day 28 onwards. This coincides with the period from suture resorption (14 days) and the subsequent inflammatory reduction (a further 14 days). From this point in spinal CCI there is a gradual recovery of axon numbers and also a decrease in neuropathic pain behaviours seen in the animals (Guilbaud et al, 1993). The time-course of these morphological changes within the injured nerve has been studied alongside the behavioural changes which develop. These studies have revealed that the onset of hyperalgesia is generally maximal at days 10-14 post-operatively (Attal et al, 1990; Bennett and Xie, 1988), the time point which corresponds to the first degenerative stage (Basbaum,1991; Coggeshall, 1993). Guilbaud et al found that behavioural signs of thermal hyperalgesia and mechanical allodynia were maximal at week 2 post-operatively in the CCI model, and progressively recovered from weeks 3-4 onwards (Guilbaud et al, 1993). At week two there is a massive Wallerian degeneration in the nerve with regeneration from week three (Ramer and Bisby, 1997), which coincides with these changes. However, the largest fibres had not recovered 15 weeks post-operatively, whereas the behavioural changes had disappeared by weeks 8-10 (Guilbaud et al, 1993). This indicates that nerve damage is important in the initiation and persistence of neuropathic pain states but there is not a strong correlation between the time-course of morphological changes (at least in large fibres) and neuropathic pain behaviours. It is likely that this is an important link in the development and

maintenance of neuropathic pain behaviours, but that additional factors also play a role.

Within the cell body of DRG neurons with injured peripheral axons, several changes also take place. When a neuron is injured the components of the cytoskeleton rearrange. Neurofilaments (NF) are thought to play an important structural role in axons and NF gene expression, NF content and axonal diameter appear closely linked through many processes of neural plasticity; development, maturation, regeneration and ageing (Scott et al, 1999). Neurofilaments are type III intermediate filaments. These filaments have highly phosphorylated side branches and when in proximity to other, similarly charged side branches of adjacent filaments act to repel them. Neurofilaments tend to become more densely packed together following injury and decrease axonal transport (Witt and Brady, 2000). This is thought to be a function of a decrease in the phosphorylation state of the neurofilaments.

During regeneration of peripheral axons, the narrowing of axonal diameter (which occurs until connections are re-established) is also accompanied by a reduction in NF synthesis and levels (Hoffman et al, 1985; Oblinger and Lasek, 1988). Sciatic nerve injury causes marked alterations in the expression of afferent cell proteins thought to be necessary for axonal regeneration; for example, reduced expression of NF proteins and increased tubulin and GAP-43 expression (Hoffman et al, 1989; Greenberg, et al, 1988; Wong et al, 1990).

A variety of multivalent docking proteins can interact with neuronal cytoskeletal elements and affect the normal morphology and functioning of these elements, and as a result ultimately the overall cellular function. One family of proteins shown to interact with such structural elements is the synuclein family of proteins.

Synuclein proteins

α and β synuclein, which are expressed strongly in the central nervous system, in particular in presynaptic terminals, have been specifically implicated in synaptic plasticity (Maroteaux and Scheller, 1991; Iwai et al, 1995). Their expression in telencephalic song-control nuclei in the Zebra Finch is closely coupled to the critical period for song learning, when major synaptic rearrangements occur (Clayton and

George, 1998). Their close relative, γ-synuclein/persyn, is expressed at high levels in spinal cord (Buchman et al, 1998b) and could also potentially influence the central synaptic plasticity that results from CCI.

Although re-normalisation of sensory function appears to occur within several weeks after sciatic CCI, the molecular mechanisms underlying this resolution remain unknown. It has been suggested however, that the time-course of the nociceptive disorders may be related to that of the axonal degeneration/regeneration process (Coggeshall et al, 1993; Guilbaud et al, 1993; Filliatreau et al, 1994) rather than the levels and pattern of particular neuropeptide expression (Munglani et al, 1996). Recovery of axonal transport following CCI has been suggested to reflect a process of efficient re-elongation of injured axons and re-connection of their targets, a process requiring re-modelling of NF networks in the regenerating axon, (Filliatreau et al, 1994).

Interestingly, the synuclein family member γ-synuclein/persyn, (expressed selectively in the peripheral nervous system) is represented at its highest levels in DRG and especially trigeminal ganglia (Buchman et al, 1998a and 1998b). γ-synuclein/persyn over-expression increases the susceptibility of the NF protein, NF-H (heavy chain) to proteolysis by calcium-dependent proteases (Buchman et al, 1998b), whereas NF-L (light chain) and NF-M (medium chain) levels are unaffected. Assembly of new NF networks is likely to be disrupted by γ-synuclein/persyn, while peptide fragments from NF-H degradation may promote disintegration of existing arrays (Wong et al, 1990a). In vivo, such effects are likely to be greatest at the periphery of the cell body, axons and processes, where γ-synuclein/persyn is predominantly localised (Buchman et al, 1998b). Peripheral axotomy of sensory neurons also results in the delayed aberrant phosphorylation of NF proteins (Goldstein et al, 1987), which is thought to result in altered NF organisation and may contribute to sensory neurodegenerative changes (Fernyhough et al, 1999), although nothing is yet known of the ways in which γ-synuclein/persyn may contribute to influencing these events. Cellular processes requiring the integrity of NF for their maintenance or cellular changes such as responses to injury, regeneration and growth, which may require turnover of neurofilament proteins, could well be influenced by γ -synuclein/persyn.

1.10.2 Phenotypic Changes in sensory nerves following peripheral sensory nerve injury

Changes in the protein expression profile of afferent neurons are seen following trauma. Expression of SP, CGRP and somatostatin is down-regulated in afferent C-fibres and small A-δ fibres following injury whereas galanin, VIP and PACAP increase (Hokfelt, et al, 1994; Zhang, et al, 1996). Galanin is strongly up-regulated in small and medium diameter neurons (Hokfelt et al, 1987; Villar et al, 1989), especially those that normally contain substance P and CGRP (Doughty et al, 1991; Kashiba et al, 1992), the majority of which are unmyelinated. Studies of galanin knockout mice have suggested that this up-regulation may be important for the development of central sensitisation (Wynick et al, 2001) and intrathecal application of galanin has been shown to increase behavioural reflex sensitivity in naïve animals (Kerr et al, 2000). The increase in VIP and PACAP occurring as a response to nerve injury, implies that these peptides could also be important in the mediation of neuropathic pain. In large diameter neurons in the DRG, levels of CGRP and SOM are again reduced whereas, NPY, VIP, galanin and CCK markedly increase after axotomy and CCI (Hokfelt et al, 1994; Nahin, et al, 1994).

Importantly, it seems that A-β fibres undergo a phenotypic switch following axotomy and begin to express SP where normally they do not (although small diameter cells decrease SP expression resulting in an overall decrease). Furthermore following SNL injury, normal low threshold input to the large fibres may induce the release of SP in the dorsal horn and contribute to pain mechanisms that would otherwise only be seen in response to nociceptive signals in the smaller fibres (Malcangio et al, 2000; Noguchi et al 1995). These changes are consistent between various models of spinal nerve damage, however in the trigeminal system, these changes are dependent on the site of injury. A transection of the full mandibular branch of the trigeminal nerve in the rat gives rise to very similar changes as shown spinally (Kar and Quirion, 1992; Nahin et al, 1994; Zhang et al, 1996), whereas in the ferret, a section of the inferior alveolar nerve produces a decrease in SP and CGRP but no change in the expression of galanin, NPY or VIP (Elcock et al, 2001).

Substance P (SP)

Following axotomy there is a significant decrease in the expression of SP mRNA in affected small diameter DRG cells, which is most pronounced at day 10-14 PO and is accompanied by a parallel decrease in SP levels in the spinal dorsal horn (Barbut et al, 1981; Jessel et al. 1979; Noguchi et al. 1993; Shehab and Atkinson, 1986b). A similar marked decrease in SP production by primary afferent neurons is seen following CCI (Cameron et al. 1991; 1997; Nahin et al.1994), but not as much as after axotomy. This is probably due to less destruction of small diameter fibres seen in CCI than in axotomy. However, there is evidence for an increase in SP in larger Aβ-fibres cell bodies in the DRG following SNL injury (Malcangio et al, 2000). Of the three SP receptors, NK_{1-3}, the NK_1 receptor is most highly expressed in the dorsal horn (Yashpal et al, 1990) but all three are up regulated in the spinal cord following nerve injury (Yashpal et al, 1991). Several studies have shown that NK_1 antagonists attenuate hyperalgesia associated with nerve damage but not mechanical allodynia (Coudore-Civiale et al, 1998; Gonzalez et al, 2000; Cahill and Coderre, 2002). NK_1 knockout mice studies also support these findings (Mansikka et al, 2000). Unfortunately however, to date NK_1 receptor antagonists have failed to show any marked analgesic activity in clinical trials in spite of this abundant evidence from laboratory research. It has been suggested that NK_1 antagonists may be mediating behaviour via their actions on supraspinal receptors and modulating the behavioural response to stress (Hill, 2000). Interestingly however, there is evidence that the NK_1 antagonist CP99994 does have a beneficial effect on dental pain (Dionne, 1999).

CGRP

Following chronic constriction of the sciatic nerve, approximately a 50% reduction in the levels of CGRP mRNA in the DRG has been reported within 7-14 days following nerve injury (Nahin et al. 1994). However, some groups found that decreases in the immunohistochemical staining for this peptide in the spinal dorsal horn were not observed until approximately 60 days after the chronic constriction injury (Kajander and Xu, 1995), while others have reported large decreases at 14 days (Carlton and Coggeshall, 1996). This disparity is possibly due to differences in nerve injury models used or species difference between these experiments.

CCK

CCK is present in relatively low amounts in DRG normally (Fuji et al, 1985; Ju et al, 1987). The role of CCK in chronic neuropathic pain states is not clear (Wiesenfeld-Hallin and Xu, 1996a), but it appears to decrease the analgesic effect of morphine and β-endorphin (Faris et al, 1983). In addition, antagonists of the CCK-B receptor (which is the prevalent form of CCK receptor within the rat spinal cord) potentiate the effects of opioid analgesic drugs and may prevent the development of morphine tolerance (Baber et al, 1989; Dourish et al, 1990; Weisenfeld-Hallin et al, 1990d). This interactive role of CCK, and the fact that its levels are dramatically up-regulated in primary afferent neurons following nerve injury (Villar et al, 1989) may therefore help to explain the decreased efficacy of opioid treatment commonly observed in neuropathic patients.

Galanin

After nerve injury, changes seen in galanin expression are variable. In the rat, sciatic nerve damage produces a large up-regulation of galanin expression in the DRG (Hokfelt et al, 1987; Kashiba et al, 1992;Villar et al, 1989). However in the trigeminal system, sectioning of the inferior alveolar nerve (part of the mandibular branch of the trigeminal nerve) in the ferret resulted in a reduction of galanin expression in the trigeminal ganglion (Elcock et al, 2001). Furthermore, transection of the infraorbital nerve in adult rats and examination of the brainstem 7 days later indicated marked reductions in the density of galanin binding sites in all regions of the trigeminal brainstem complex (Bodie et al, 1997). Galanin is known to inhibit transmission in the dorsal horn (Hokfelt et al, 1987; Wiesenfeld-Hallin et al, 1989; Yanagisawa et al, 1986) and has been shown to have analgesic actions (Post et al, 1988; Villar et al, 1991). It seems likely that these effects would be enhanced following sciatic nerve injury, but these results suggest that it is unlikely that galanin has this role following injury to the ferret IAN.

The pattern of co-expression of galanin and the other neuropeptides also changes following nerve injury with less CGRP/galanin co-expression and an increase in the co-existence of galanin with NPY and VIP (Nahin et al, 1994).

NPY

Following nerve injury the local administration of a Y2 receptor agonist in the injured paw increased mechanical and thermal hyperalgesia. Similar administration of the Y1/Y5 agonist [Leu31, Pro34]-NPY increased mechanical hyperalgesia but decreased thermal hyperalgesia (Tracey et al 1995). However, in another study using a similar model, intrathecal administration of NPY also exacerbated nerve injury-induced hyperalgesia (White, 1997), but [Leu31, Pro34]-NPY enhanced hyperalgesia and N-acetyl[Leu28,31]NPY24 – 36 had no effect. Interestingly, Y1-KO mice exhibit a hyperalgesic phenotype in neuropathic nociception experiments (partial nerve injury) (Naveilhan et al, 2001).

Following nerve injury, NPY expression markedly increases in DRG cells, especially those with large diameter axons (Hokfelt et al, 1994; McMahon and Priestley, 1995), and axotomy of the inferior alveolar nerve in the rat has been shown to cause an increase in NPY-immunoreactivity in the SpC and TG (Sasaki et al, 1994). Two other rat studies have reported a marked up-regulation of NPY expression following trigeminal nerve injury (Fristad et al, 1996; Wakisaka et al, 1993). In the ferret however, few cells were seen to express NPY in the trigeminal ganglion, with no change after injury (Elcock et al, 2001). These results may differ due to species, but also, more severe changes are reported when the injury is closer to the nerve cell bodies (Lieberman, 1974) therefore site of nerve injury may be important. NPY is known to play a role in modulating nociceptive inputs (Hua et al, 1991; Munglani et al, 1996) and is believed to have an analgesic role. Thus, the increase in expression reported in previous studies may indicate a mechanism for a reduction in nociceptive input occurring as a consequence of nerve injury. However, this is unlikely to play such a role in the ferret following IAN injury.

VIP/PACAP

VIP expression is dramatically up-regulated in the spinal dorsal horn (Shehab and Atkinson, 1986) and in the trigeminal ganglion and trigeminal complex Atkinson and Shehab, 1986; Fristad et al, 1998) following nerve injury. Yet it has also been shown to remain unchanged in the trigeminal ganglion in the ferret (Elcock et al, 2001).

Dorsal rhizotomy produces an almost total depletion of VIP from the LI of the dorsal horn, indicating that the origin of VIP is in the fine primary afferent fibres (Shehab and Atkinson, 1986; Yaksh et al, 1982). As a result of nerve injury, levels of PACAP

a member of the same family of peptides, increases in DRG neurons within 1-2 days and then slowly subsides (Zhang et al, 1995). VIP levels, however, gradually increase in the first two weeks, but stay increased for long periods of time (Nahin et al, 1994). It is this which has lead to the assertion that PACAP may be involved in the early onset of neuropathic pain and that VIP may be important in maintaining neuropathic pain states. In fact, in an experiment using agonists and antagonists to substance P and CGRP, following nerve transection substance P and CGRP were no longer excitatory, whereas VIP was (Wiesenfeld-Hallin et al, 1991). These results may suggest that the role of tachykinins in mediating C-afferent- induced reflex facilitation is taken over by VIP after axotomy. This coupled with the pattern of change in the expression of these peptides, suggests that there may be a functional exchange from SP to VIP in nociceptive processing in the case of nerve injury.

The actions of VIP and its close homologue PACAP are mediated through three G-protein coupled receptors PAC_1 (which has a higher affinity for PACAP than VIP), $VPAC_1$ and $VPAC_2$ (which have similar affinities for VIP and PACAP) (Ishihara et al, 1992; Lutz et al, 1993; Hosoya et al, 1993). Of these only the $VPAC_2$ receptor is increased in expression in the spinal cord following nerve injury while $VPAC_1$ is decreased and PAC_1 is unaltered (Inagaki et al, 1994; Dickinson et al, 1999). Predictably, neuronal excitation by an ionophoretically applied $VPAC_1$ receptor agonist is reduced after nerve injury and that to a $VPAC_2$ receptor agonist is increased (Dickinson et al, 1999). In neuropathic animals only PAC_1 and $VPAC_1$ antagonists had any effect on cold-induced neuronal firing activity, however, antagonists for all three receptors, but particularly $VPAC_2$, inhibited mustard oil-induced activity in neuropathic animals (Dickinson et al, 1999).

Additional factors affecting neuropeptide plasticity.

There is a marked change in DRG gene expression after nerve damage, and several different mechanisms may be involved. In the case of VIP, there is strong evidence that the regulation of VIP expression in DRG is mediated by factor(s) carried by retrograde axoplasmic transport to DRG cell bodies as axotomy or nerve crush produce increased VIP levels which return to control values after nerve regeneration (Knyihar-Csillik et al. 1991; 1993).

VIP immunoreactivity in the upper spinal dorsal horn (largely of afferent origin) is markedly increased after distal transection or crush treatment of the ipsilateral

peripheral nerve (Shehab and Atkinson, 1986; Yaksh et al, 1982). After peripheral nerve regeneration, VIP disappears from the upper dorsal horn. Transection-induced VIP increase is abolished by dorsal rhizotomy. It is concluded that the expression of VIP is restricted by factor(s) carried by retrograde axoplasmic transport to dorsal root ganglion cells (Knyihar-Csillik et al, 1991). It appears that blockade of the retrograde axoplasmic transport induces a switch in the gene expression of dorsal root ganglion cells, which results in the expression of VIP instead of substance P and CGRP (Wiesenfeld-Hallin et al, 1990). Nerve injury also alters expression of neurotrophins and their receptors in the DRG neurons, which include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and leukaemia inhibitory factor (LIF) (Cameron et al, 1997; Nahin et al, 1994; Sebert, 1993).

The expression of SP by DRG neurons appears to be dependent on the availability of NGF (Lindsay, 1989). Intrathecal NGF has been shown to prevent the axotomy-induced decreases in levels of CGRP and SP (Fitzgerald et al, 1985), while application of anti-NGF augmented the change in CGRP and SP levels (Fitzgerald 1985). It is possible that nerve injury interrupts the retrograde transport of NGF, and this may directly or indirectly influence CGRP and SP levels. Conversely, VIP expression in adult DRG neurons in culture appears to be independent of NGF (Mulderry, 1990).

Figure 1.4

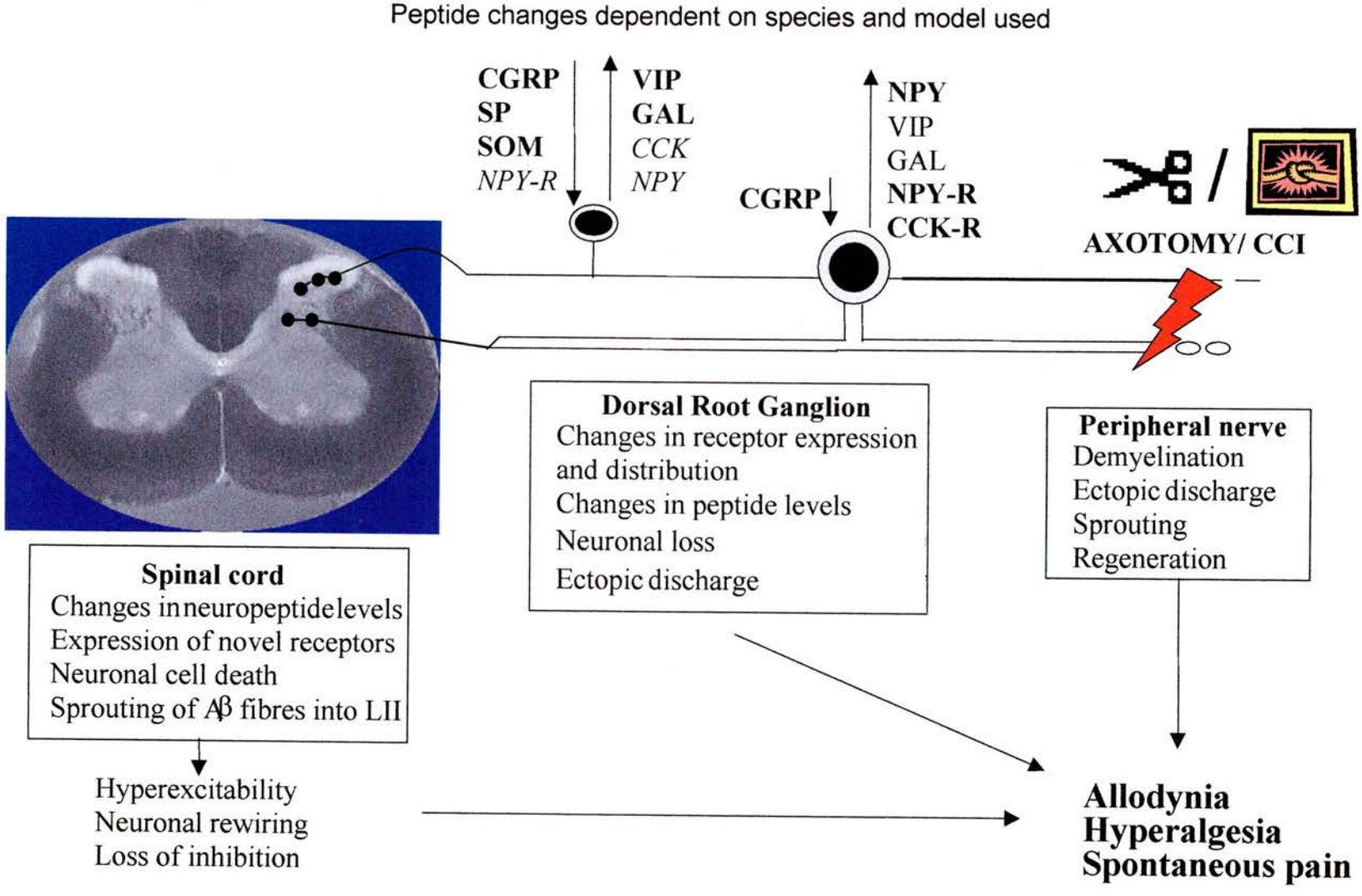
Schematic of the Principal Changes within the CNS Following Peripheral Nerve Injury

Schematic drawing of small and large primary sensory neurones in a ganglion (in this case a dorsal root ganglion sending a central branch to the dorsal horn of the spinal cord), with a peripheral branch which has undergone axotomy or chronic constriction injury. The main changes which occur within the central nervous system are highlighted although all of these changes are dependant on the model of injury used and the species of animal.

The changes in levels of peptides and their receptors are indicated by arrows. Thus in small diameter DRG neurones the level of substance P (SP), calcitonin gene-related peptide (CGRP) and somatostatin (SOM) are decreased, along with neuropeptide Y (NPY) receptor mRNA. In contrast levels of vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP) and galanin (GAL) are markedly increased, with modest increase in cholecystokinin (CCK) and NPY. In the large diameter DRG there is a prominent increase in the levels of NPY and the NPY receptor, along with smaller increases in VIP and GAL, while the expression of CGRP is seen to decrease.

Structural and neurochemical reorganisation within the spinal cord and the damaged nerve, as indicated, ultimately contribute to the development of spontaneous ectopic firing in A fibres and an increased hyperexcitability of C fibres, as well as providing the potential for the development of abnormal A fibre responses, including a loss of inhibitory properties.

(Adapted from Hokfelt et al. 1994)


Peptide changes dependent on species and model used
CGRP
SP
SOM
NPY-R
VIP
GAL
CCK
NPY
CGRP
NPY
VIP
GAL
NPY-R
CCK-R
AXOTOMY/ CCI
Dorsal Root Ganglion
Changes in receptor expression and distribution
Changes in peptide levels
Neuronal loss
Ectopic discharge
Peripheral nerve
Demyelination
Ectopic discharge
Sprouting
Regeneration
Spinal cord
Changes in neuropeptide levels
Expression of novel receptors
Neuronal cell death
Sprouting of Aβ fibres into LII
Hyperexcitability
Neuronal rewiring
Loss of inhibition
Allodynia
Hyperalgesia
Spontaneous pain

1.10.3 Changes in the initial relays of the spinal cord or trigeminal nucleus of the Central Nervous System.

Changes in the peripheral nerve, such as the appearance of ectopic discharges, are thought to induce changes in the dorsal horn of the spinal cord. This can give rise to the phenomenon of central sensitisation resulting in an increased response to, and changes in, the processing of afferent input (Woolf and Mannion, 1999).

The phenomenon of central sensitisation is thought to cause a number of changes that can be seen in the behavioural responses to stimuli.

They are;

1. Enlargement of the receptive field, (the area in the periphery where a stimulus will activate neurons).
2. Increased response to a supra-threshold input; and
3. Previously sub-threshold inputs reaching threshold and initiating action potential discharge.

These changes are manifest as hypersensitivity to pain that spreads from the site of injury (primary and secondary hyperalgesia) as well as pain-like responses to previously innocuous stimulation (mechanical and thermal allodynia).

Spinal transmission can be modulated from supraspinal sites, which exert both facilitatory and inhibitory influences (Urban et al 1999). The net balance of these multiple supraspinal pathways, together with primary afferent input, will ultimately influence the excitability of spinal neurones (Kauppila et al, 1998). There is increasing evidence for a significant role of supraspinal excitatory influences in the development and maintenance of central sensitisation in persistent pain states, including peripheral nerve injury (Ossipovet al 2001;, Porreca et al, 2002; and Urban et al 1999). In particular, studies have implicated the rostral ventral medial medulla (RVM), which contains the raphe magnus nucleus (RMg), together with other nuclei, such as nucleus reticularis gigantocellularis (NGC) and nucleus reticularis gigantocellularis pars alpha (NGFα). These structures are involved in the bulbar relay of descending modulatory projections to the spinal cord (Calejesan et al, 1998;, and Zhuo and Gebhart, 1990b) and electrical stimulation of RVM has been shown to produce enhanced behavioural nociceptive reflexes (Zhuo and Gebhart, 1990a; 1990b and 1991). Furthermore, inactivation of RVM or spinal transection suppresses

mechanical allodynia following peripheral nerve injury (Bian et al, 1998; Kauppila et al, 1998 and Sung et al, 1998).

Several changes take place in the central nervous system, that are thought to underpin this sensitisation. One of the most crucial is the involvement of the NMDA glutamate receptor and docking proteins associated with it in a functional multiprotein complex.

The involvement of the NMDA receptor and its docking protein complex in central sensitisation

Central sensitisation has been demonstrated in both the spinal cord and trigeminal system and has been shown to be dependent on activation of the NMDA receptor. It has also been shown that antagonists to the NMDA receptor inhibit the development of central sensitisation in the spinal dorsal horn and trigeminal nuclei (Davis and Lodge, 1987; Dickenson and Sullivan, 1987; Parada et al 1997; Chiang et al, 1998; Park et al, 2001; Yonehara et al 2003; Woolf and Thompson, 1991; Boyce et al 1999). NMDARs are composed of NR1, NR2 (A, B, C, and D) and NR3 (A and B) subunits. Co-expression studies have demonstrated that formation of functional NMDAR channels requires a combination of NR1, an essential channel-forming subunit, and at least one of the NR2 subunits (Mori and Mishina, 1995). It is bi-ligand gated and is activated by binding both glutamate and glycine. Site-directed mutagenesis and molecular modelling studies have disclosed critical determinants of the glutamate and glycine binding sites and demonstrated that they are located on the homologous regions of the NR2 and NR1 subunits, respectively (Hirai et al, 1996; Laube et al 1997). Usually this receptor channel is blocked by magnesium ions but when activated it allows the conductance of calcium. An NMDA receptor-mediated increase in intracellular calcium will elicit an array of intracellular changes including the activation of calcium-dependent kinases, phosphatases and other proteins which may lead to both acute organisational and longer term genomic changes in neurons (Sigel, 1995; Lledo et al, 1995).

Co-expression studies have also shown that many biophysical and pharmacological properties of the heteromeric NR1/NR2 NMDAR channels, such as sensitivity to

magnesium block, kinetics of desensitization and offset decay, susceptibility to modulation by glycine, reducing agents, polyamines and phosphorylation, and affinity for agonists and antagonists, depend on the type of NR2 subunit included in a heteromeric complex (Yamakura and Shimoji, 1999; Cull-Candy et al, 2001). For example, a brief application of glutamate onto NR1/NR2A assemblies generates a macroscopic current with a deactivation time constant of tens of milliseconds, compared with several seconds for NR1/NR2D receptors (Yamakura and Shimoji, 1999; Cull-Candy et al, 2001). In addition, NR1/NR2A assemblies are less sensitive to glutamate than other heteromeric channels. Aside from these kinetic differences, the most obvious subunit-dependent properties of NMDARs are their single-channel conductance and sensitivity to magnesium block. For example, the NR2A or NR2B subunits-containing NMDARs generate high-conductance channel openings with a high sensitivity for blocking by magnesium, whereas NR2C- or NR2D-containing receptors give rise to low-conductance openings with a lower sensitivity to magnesium. NR3 subunits do not by themselves generate agonist-activated currents. In fact when co-expressed with NR1 and NR2, NR3A/3B containing NMDARs act in a dominant-negative manner against NMDARs to reduce NMDA-induced currents (Ciabarra et al, 1995; Sucher et al, 1995; Nishi et al, 2001). In contrast to conventional NR1/NR2 receptors, co-assembly of NR1 with NR3A or -3B subunits forms excitatory glycine receptors that are unaffected by glutamate or NMDA, are impermeable to calcium, and are resistant to magnesium block and NMDAR antagonists (Chatterton et al, 2002). To date no experimental studies related to the role of NR3 subunits in pain mechanisms have been performed. Therefore, this thesis will concentrate on the contribution of NMDA receptors comprised of NR1 and NR2 subunits to the role of NMDAR in sensitisation.

Many peripheral nerve fibres contain the excitatory amino acid glutamate, but importantly, there is also co-expression of peptides such as substance P in some of these fibres. As discussed in section 1.7.1, both spinal neurons and primary afferents have several types of receptors for glutamate, AMPA receptors, metabotropic receptors, kainate receptors and NMDA receptors. The AMPA receptor seems to be activated in fast transmission of acute noxious stimuli, however if the painful stimulus is continued, it is possible that peptides and glutamate may become released in

excessive amounts and start to accumulate in the synapse and surrounding area and act upon extra-synaptic receptors to increase the number of depolarisations.

Protein phosphorylation is a major mechanism for the regulation of NMDAR function. Indeed, direct phosphorylation may be one mechanism by which PKC regulates the function of NMDARs (Liao et al, 2001). Kinase activation by G-protein coupled metabotropic receptors (Bleakman et al, 1992; Chen and Huang, 1992; Bond and Lodge, 1995) is believed to phosphorylate the NMDA receptor producing a conformational change which diminishes Mg^{2+} blockade, allowing channelling of Ca^{2+} through the receptor. Furthermore, actual coupling of the NMDA receptor and metabotropic glutamate receptors mediating the effects of phosphorylation has been demonstrated (Tu et al, 1999). Also, PKC potentiates NMDA responses indirectly by activation of the tyrosine kinase (Src) signalling cascade (Lu et al, 1999). In addition, increasing evidence suggests that PKC modulates the function of NMDARs by participating in their interactions with postsynaptic density and cytoskeletal proteins (Sheng and Pak, 2000).

Although PKC enhances NMDA-evoked currents in most preparations, its effects on the magnesium blockade of NMDA currents vary (Petrenko et al, 2003). One obvious factor that can contribute to the dissimilar PKC effects found among the various studies is the differential NMDAR subunit expression in the CNS. Compared with NR1/NR2C and NR1/NR2D receptors, NR1/NR2A and NR1/NR2B receptors are more sensitive to magnesium block and PKC potentiation. Thus, consistent with the larger expressions of NR2B and NR2A subunits in the superficial dorsal horn, PKC resulted in magnesium-dependent potentiation of NMDAR-mediated responses in rat dorsal horn neurons that receive sensory inputs from an inflamed hind paw (Guo and Huang, 2001).

Another protein that is likely to mediate many aspects of postsynaptic signalling by NMDAR is calcium-calmodulin-dependent protein kinase II (CaMKII). It has received much attention because it is persistently activated after NMDAR stimulation. The activation of CaMKII stimulates its binding to the cytoplasmic domain of the NMDAR subunit NR2B. By interfering with auto-inhibitory interactions within CaMKII, binding to NR2B locks CaMKII in an activated state that cannot be reversed

by phosphatases (Bayer et al, 2001). In addition, the CaMKII-NR2B interaction leads to the trapping of CaM that may reduce down-regulation of NMDA receptor activity. CaMKII is up-regulated in the superficial laminae of the dorsal horn and DRG cells after injuries to peripheral tissues (Fang et al, 2002). Thus, the ability of CaMKII to specifically interact with NR2B, together with their co-localization in the superficial dorsal horn (a region strongly involved in nociception), indicates the particular importance of the NR2B-CaMKII interaction in the development and maintenance of nociceptive hypersensitivity and provides a novel potential target for treatment of chronic pain. This interaction of CaMKII with NMDA receptors is disrupted the spinal dorsal horn in mice with a truncated form of the scaffold protein PSD-95 (Garry et al, 2003). In addition, protein kinase A (PKA) may be an important regulator of the NMDA receptor since it is associated with the NR1 subunit via the AKAP protein Yotiao, and also to NR2 subunits via AKAP-79 and PSD-95 (Westphal et al, 1999; Colledge et al, 2000).

The NMDA receptor forms complexes with multivalent adapter proteins (Husi et al, 2000a). In fact this complex has been shown to contain in excess of 70 proteins, including several known intracellular signalling molecules (Husi et al, 2000b). Of the multivalent adapter proteins involved in the complex, one is the protein PSD-95 (Post-Synaptic Density 95kDa). This protein is one member of a family of related proteins termed the membrane associated guanylate kinases (MAGUKs). It is an abundant scaffold protein and it binds to the cytoplasmic C-terminal tails of NR2 subunits of the NMDA receptor (Husi et al, 2000b). It is crucial because it links several of the intracellular signalling pathways with the NMDA receptor (Kim et al, 1998; Chen et al 1998; Westphal et al, 1999; Colledge et al, 2000). It has been shown to be a necessary link in the SynGap-Ras-MAPK signalling pathway (Grant and O'Dell, 2001). These proteins that are linked by MAGUKs to the NMDA R, such as neuronal NO synthase, SynGAP, and SPAR, may participate in downstream signalling by NMDARs (Sheng and Kim, 2002). Not surprisingly, the NMDAR-PSD-95 interaction has been recently implicated in the processing of spinal nociceptive information. PSD-95 mRNA and protein are enriched in the spinal cord and are selectively distributed in the superficial dorsal horn neurons, where PSD-95 interacts with NR2A/2B subunits (Tao et al, 2000). Additionally, PSD-95 is required for NMDAR-mediated thermal hyperalgesia. Furthermore, PSD-95 knockdown can delay

the onset of mechanical and thermal hyperalgesia in chronic neuropathic pain model involving spinal nerves (Tao et al, 2001). Recently it has been shown that mice expressing a truncated form of the PSD-95 protein did not develop pain behaviours associated with sciatic nerve injury but did have normal response following intradermal injection of the inflammatory agent formalin to the hind paw (Garry et al, 2003). Other proteins included in the MAGUK family are SAP-97, SAP-102 and Chapsyn-110 (PSD-93) and the function of these proteins is a source of active investigation.

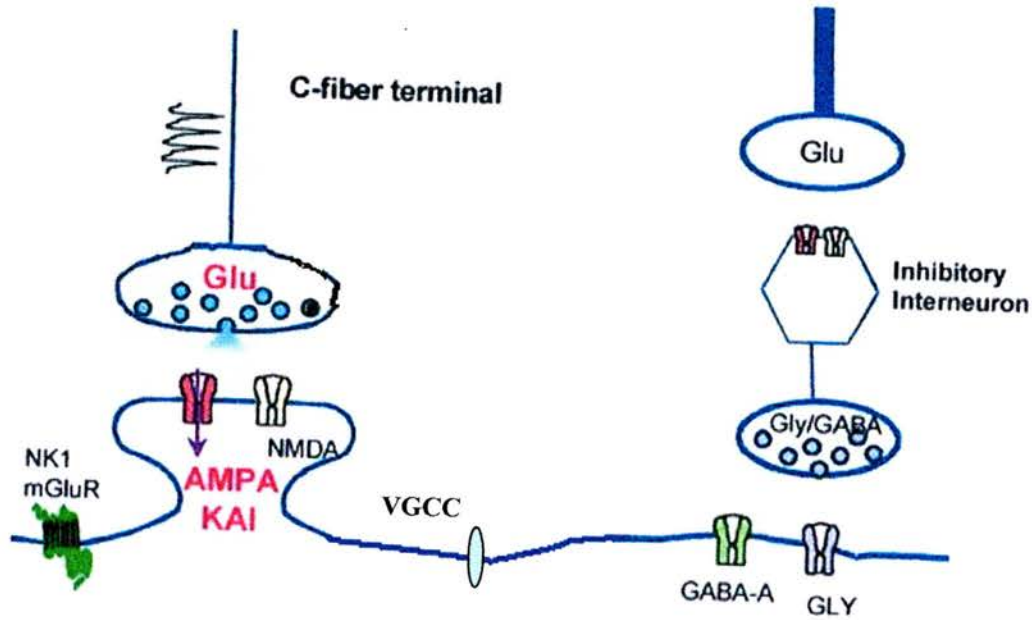
There has been much interest in NMDA receptor antagonists for their potential therapeutic role in pain states and there is considerable evidence for their effectiveness pre-clinically (Ren, 1994; Dickenson et al, 1997; Sandkuhler and Liu, 1998; Boyce et al, 1999; Suzuki et al, 2001). However, many of these antagonists have shown unacceptable side effect profiles (Yenari et al, 1998), although this appears to be less problematic with memantine (Parsons et al, 1999).
NMDA receptor activation has also been shown to be important in trigeminal central sensitisation in both the oralis (Parada et al, 1997;Park et al, 2001; Woda et al, 2001) and caudalis regions (Chiang et al, 1998; Luccarini et al, 2001). Interestingly though, NMDA subunits are differentially expressed in the trigeminal system with NR2A being present in both oralis and caudalis but NR2B being present only in the caudalis (Watanabe et al, 1994).

Figure 1.5

Post-Synaptic Integration of Signals in Gating the Role of NMDA Receptors

Modes of neural plasticity at synapses onto nociceptive transmission neurons. Modulation through intracellular kinase/phosphatase signaling cascades produces central sensitization through facilitating AMPA/kainate and NMDA receptor function or cell-surface expression. Modification is mediated by loss of inhibitory interneurons, A) In normal transmission glutamate acts on receptors and ion channels in the central nervous system to elicit Na2+ / Ca2+ entry and / or activate protein kinases that phosphorylate membrane bound NMDA and AMPA receptors and alter their functional properties. B) These processes then act to prime the NMDA receptor by removal of the Mg2+ channel block and allow further Na+ and Ca2+ influx to enhance membrane excitability and Ca2+-dependent intracellular responses, which are likely to play a key role in central sensitisation (from Woolf and Salter, 2000).

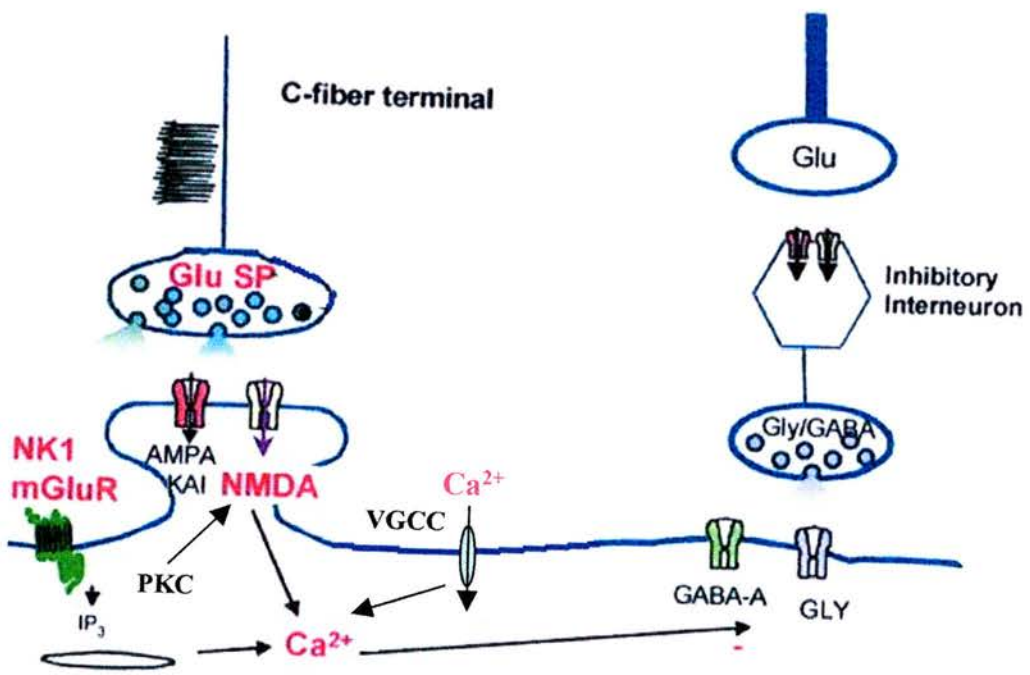
A)



Nociceptive
Dorsal horn neuron

AMPA/KAI receptor fast EPSPs

B)



Activation :
Slow EPSPs, plateau potentials, summation & windup

1.11 Summary of introduction

It has been reported for sometime now that neuropathic pain conditions of the trigeminal region have different characteristics from those seen in the spinal cord. Trigeminal neuralgia (a potential painful spasm-like condition induced by light touch to a trigger zone in the trigeminal region and having a period of latency between trigger and spasm) has no counterpart in the limbs or trunk. Causalgia (sympathetic dystrophy) occurs more frequently in the limbs than the trigeminal region (Hoffman and Matthews, 1990). Phantom limb sensations are reported in the majority of amputees often with chronic pain (Sherman et al, 1984) while phantoms and chronic neuropathic pains are extremely rare after tooth extraction even when multiple extractions are performed (Pollman 1981; Reisner 1981).

These disparate clinical findings may be underpinned by differences that exist between the spinal sensory nerve relays and those in the trigeminal sensory system. These differences range from embryonic origin of cells that form the primary afferents of the two systems, to anatomical connections between primary afferents and cells in the central nervous system and expression of receptor subunits known to participate in neuronal plasticity changes.

Because of these differences, we have investigated the electrophysiological responses within these two regions in order to compare and contrast responses following nerve injury in peripheral nerves innervating these two regions. We have also undertaken an investigation into the expression of subunits of key receptors involved in the phenomenon of central sensitisation, and all of their associated MAGUK proteins within the trigeminal oralis, caudalis and spinal cord. We have done this in naïve and neuropathic animals in order to gain an insight into the roles that these adapter proteins might play in the central sensitisation of these regions. Lastly, we have examined the contribution to chronic neuropathic pain states made by members of the synuclein family of proteins known to interact with the cytoskeletal elements. These may possibly affect the transport of mediators along axons or the interaction of the cytoskeleton with MAGUK multivalent docking proteins and their subsequent interaction with key receptors involved in synaptic plasticity.

1.12 Aims

Since neuropathic pain from trigeminal nerve injury shows different characteristics from that due to spinal nerve injury, both clinically and in animal models, we will investigate whether there are key mechanistic differences between laboratory models of these pain states. We also intend to examine the role of proteins proposed to affect key aspects of cytoskeletal change in the injured neuron. With these in mind our three aims are to;

1) Monitor neuropathic sensitisation as nociceptive reflex behaviours and electrophysiological responses of single neurons in spinal and trigeminal systems.

2) Measure chemical markers of neuropathic change (in both spinal and trigeminal regions) and investigate the transmitter and intracellular mediators underlying these changes.

3) Explore the role of synuclein proteins in degenerative and regenerative changes following nerve injury.

Chapter 2

Materials

All reagents used were of the highest analytical grade and were supplied by Sigma Chemical Company, Poole, UK, unless otherwise stated.

2.1 Anaesthetics

- Urethane, (Zeneca Ltd., Cheshire, UK)
- α-chloralose, (Zeneca Ltd., Cheshire, UK)
- Halothane (Zeneca Ltd., Cheshire, UK),
- Sagatal (Rhône Mérieux Ltd., Essex, UK)

2.2 Sterile Surgery

- 4/0 and 5/0 sterile chromic catgut and 4/0 violet coated vicryl (Ethicon Ltd., Edinburgh, UK)
- Hibitane (Zeneca Ltd., Cheshire, UK)
- Sterile gowns, biogel gloves, hats, face masks and drapes (Hospital Management and Supplies, Glasgow, UK)

2.3 Electrophysiology

- Glass capillaries (Clark Electromedical Ltd., Reading, UK)
- Platinum wire (Goodfellow Metals, Cambridge, UK)
- Liquid Paraffin (Thornton and Ross, Huddersfield, UK)
- Agar (Unipath Ltd., Basingstoke, UK)
- Axoprobe 1-A amplifier (Axon Instruments, CA, USA)
- Digitimer (D100, Digitimer Ltd Welwyn Garden City, Herts UK)
- Dual beam oscilloscope (D13, Tektronix Inc., Beaverton, OR, USA),
- Spike processor (D.130, Digitimer, UK)
- Standard 1401 data acquisition unit (CED, Cambridge, UK)
- Spike 2 software (CED, Cambridge, UK)

2.4 Histological marking experiments

- Isopentane (Merck Ltd., Leics, UK)
- Pontamine Sky Blue (Merck-BDH, UK)
- Cryostat (Bright instruments, UK)

2.5 Western Blot Apparatus

- 10% Precast Tris-HCl ready gels (10% resolving gel, 4% stacking gel, Bio-Rad)
- Immuno-blot PVDF membrane (Bio-Rad)
- Methanol (Hayman)
- Blot absorbent filter paper (thick) (Bio-Rad)
- Glacial acetic acid (Sigma)
- Polyoxyethylenesorbitan monolaurate (Tween-20, Sigma)
- Lauryl sulphate (SDS, Sigma)
- Enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham)
- Hyperfilm (ECL) autoradiography film (Amersham)

2.6 Development of Animal Models

- 1ml syringe (Merck-BDH, UK)
- 25Gx1 needle (Terumo, Belgium)
- Coltene Labputty (Henry Schein, UK)

2.7 Behavioural Reflex Testing

- Thermal stimulus (Hargreaves-Plantar apparatus, Ugo Basile, Italy).
- Von Frey filaments (Stoelting Co., Wood Dale, Illinois).
- Acetone (Zeneca Ltd., Cheshire, UK)

2.8 Antibodies

Primary concentration

- Anti PSD-95 (Upstate biotech, USA) 1:750
- Anti NR1 (Santa Cruz, biotech, Inc, USA) 1:100
- Anti NR2A (Upstate biotech, USA) 1:100
- Anti NR2B (Chemicon International, CA, USA) 1:100
- Anti SAP 97 (Santa Cruz, biotech, Inc, USA) 1:200
- Anti SAP 102 (Chemicon International, CA, USA) 1:200
- Anti Chapsyn 110 (Chemicon International, CA, USA) 1:200
- Anti GAPDH (Chemicon International, CA, USA) 1:750
- Anti- Glur1 (Upstate biotech, USA) 1:200
- Anti-Glur2 (Chemicon International, CA, USA) 1:200

Secondary

- Goat anti-mouse-FITC (Southern Biotechnology Associates, Birmingham, Alabama) Immunohistochemistry. 1:2500
- Goat anti-mouse HRP-conjugate (Chemicon International, CA, USA) 1:2000
- Goat anti-rabbit HRP-conjugate (Chemicon International, CA, USA) 1:25000

2.9 Miscellaneous

- Freezing spray (Greenhill Chemical Products Ltd., Burton-on-Trent, UK)
- Glass coverslips (22 x 50mm) (Merck-BDH, UK)
- Silica gel (Merck-BDH, UK)
- Decon 90 (Decon Laboratories Ltd., Hove, UK)

2.10 Stock solutions

- Phosphate-buffered saline (PBS)

  50mM $NaHCO_3$, pH 7.5; 150mM NaCL

- 4% paraformaldehyde (PFA) in PBS

  0.1M PBS, 4% (w/v) (fresh) paraformaldehyde

- SDS lysis buffer (5X Stock)

  0.625M Tris-Cl, pH 6.8; 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 50% (v/v) glycerol

- Electrophoresis buffer

  1M Tris base, 0.1M glycine (+SDS)

- Coomassie staining reagent

  0.1% (w/v) Coomassie brilliant blue R-250, 25% (v/v) isopropanol, 10% (v/v) acetic acid

- Destaining reagent

  10% (v/v) acetic acid, 5% (v/v) isopropanol

- Protein transfer buffer

  0.1M Tris base, 0.5M glycine, 0.01% (w/v) SDS

- Blocking buffer

  1X PBS, 4% (w/v) non-fat milk (Marvel)

- Wash buffer

  1X PBS, 0.1% (v/v) Tween 20

Chapter 3
Methods

3.1 Chronic Constriction Injury (CCI) to the Sciatic Nerve

All experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986. Adult male Wistar rats (200-350g) were anaesthetised with halothane / O_2 (2-3%). (Figure 3.1A) Under aseptic conditions, the right sciatic nerve was exposed proximal to the trifurcation, at a mid-thigh level, and 4 (4 / 0, Ethicon) chromic cat gut ligatures were tied to loosely constrict (i.e. to constrict the nerve until the diameter of the nerve just starts to decrease, unfortunately no reliable method for calibrating ligature tightness exists) the nerve (chronic constriction injury (CCI)), as viewed under x40 magnification (Bennett and Xie, 1988). The overlying muscle and skin were closed with resorbable sutures (4 / 0 vicryl, Ethicon) and the animals were allowed to recover for 72 hours (without the need for antibiotics) before reflex testing recommenced. CCI surgery was also carried out in mice for some experiments (Chapter 4) in which case 3 (5/0) chromic catgut sutures were placed around the nerve. In the described studies using mice, all mutant and wild type mice were of the C57/Black6 strain and were operated on as described previously in Garry et al 2003. Due to greater susceptibility to inhalation anaesthetic adult male—mice were anaesthetised with Sagatal (0.01ml / 100g, i.p.) and supplemented with halothane / O_2 (0.5-1.5%) however due to the length of the operation there was no difference in the post-surgical recovery time of these two regimes. Under aseptic conditions, the right sciatic nerve was exposed proximal to the trifurcation, at the mid-thigh level, and 3 (5 / 0, Ethicon) chromic cat gut ligatures were tied to loosely constrict the nerve viewed under x40 magnification. The overlying muscle and skin were closed with sutures (4 / 0 vicryl, Ethicon) and the animals allowed to recover for 72 hours before reflex testing recommenced. Sham-operated rats and mice underwent the same surgical procedure, but no ligatures were placed around the nerve.

3.2 Chronic Constriction Injury (CCI) to the Infraorbital Nerve

For trigeminal nerve injury, adult male Wistar rats (200-350g) were anaesthetised with halothane / O_2 (2-3%) delivered through a specially constructed mouthpiece. A midline incision was made along the top of the skull and a full thickness flap of skin through to and including the periosteum was lifted and retracted to allow access to the orbital floor where the nerve is situated. The infraorbital nerve was dissected free from the surrounding connective tissue and once exposed, loosely held in specially constructed nerve retractors and 2 (5 / 0, Ethicon) chromic cat gut ligatures were tied to loosely constrict the nerve viewed under x40 magnification (Figure3.2). The overlying muscle and skin were closed with sutures (4 / 0 vicryl, Ethicon) and the animals allowed to recover for 72 hours before reflex testing recommenced. This surgery caused no disruption to the orbital contents and the position of the eye and vision appeared-normal afterwards. Sham-operated rats underwent the same surgical procedure, but no ligatures were placed around the nerve.

3.3 Behavioural Somatosensory Reflex Tests

Behavioural signs representing up to three different components of neuropathic pain were investigated. Mechanical allodynia was assessed in animals following nerve constriction injury to the sciatic or infraorbital nerves, whereas thermal hyperalgesia, and cold allodynia assessments were only carried out in animals following sciatic nerve constriction injury, due to the greater technical difficulty of performing such tests in the infraorbital model. Behavioural testing was carried out prior to surgery to establish a baseline for comparison to post-surgical values. Only nerve-injured animals that showed clear signs of thermal hyperalgesia, cold and mechanical allodynia were used for further study.

Although the models are similar in basic principle, the proportion of relevant afferents affected and the functional consequences of the lesion may well differ. The magnitude and composition of the reflex responses are also clearly different between

the spinal and trigeminal models. So results from the two models are not directly comparable, at least in absolute, quantitative terms.

3.3.1 Sciatic Nerve (CCI) Treated Animals

General Observations

Inspections were regularly made for any signs of autotomy, that is, biting or gnawing of the paw or leg. This was rarely observed and any animals that displayed signs of this behaviour were immediately euthanased. The incision site was regularly checked for signs of infection to ensure proper healing. General observation of the animals' posture and behaviour (including exploratory, feeding and grooming behaviour) were also made. All animals used in this study were found to have normal behaviours and weight gain following surgery.

Measurement of Thermal Hyperalgesia

Thermal hyperalgesia was monitored using radiant heat (30-55°C) applied to the mid-plantar glabrous surface of the hind paw using Hargreaves' thermal device. The withdrawal response latency was characterised as a brief paw flick recorded to the nearest 0.1s, and a standard cut-off latency of 20s was employed to prevent tissue damage. Testing was repeated a minimum of 5 times with not less than 5 min between trials to avoid any sensitisation of the paw.

Measurement of Mechanical Allodynia

Mechanical allodynia was measured as the threshold for paw withdrawal in response to normally innocuous graded mechanical stimuli applied to the mid-plantar glabrous surface of the hind paw using Semmes-Weinstein calibrated von Frey filaments (Stoelting) (Figure 3.1B). Each filament was applied perpendicularly to the mid-plantar surface of the foot until it started to bend. This was repeated 10 times at a frequency of approximately 1Hz. The filaments were applied in ascending order and

a response characterised as a robust paw flick. Threshold was defined as the force that caused foot withdrawal 5 times in every 10 applications.

Measurement of Cold Allodynia

To detect the presence of cold allodynia, rats were placed in a perspex box with an elevated aluminium floor covered with iced water, sufficient to immerse both glabrous and hairy skin of the hind paw (3-4°C; Bennett and Xie, 1988). Rats were allowed 10s to acclimatise once placed in the box. The number of seconds the animal raised its hind paw above the water over a 20s period was recorded. This was repeated 4 times at 10 min intervals to establish a mean suspended paw elevation time (SPET) for each rat. Animals determined as being at the peak of neuropathy, 12-15 days following CCI surgery, were then used in further study. All tests were consistent with previous experiments in CCI rats with in this laboratory showing no response to bath of water at room temp (25 degrees Celsius).

3.3.2 Trigeminal Nerve (CCIio) Treated Animals

General Observations

Inspections were regularly made for any signs of autotomy which, in the face region, may show as scratches or patches of hairlessness due to over-grooming. This was rarely observed and any animals that displayed signs of this behaviour were immediately euthanased. General observation of the animals' animals' posture and behaviour (including exploratory, feeding and grooming behaviour) were also made. All animals used in this study were found to have normal behaviours and weight gain following surgery.

Measurement of Mechanical Allodynia

Again mechanical allodynia was measured using Semmes-Weinstein calibrated von Frey filaments (Stoelting). Here, however, animals were restrained using circular

perspex tubes and the head allowed to move freely (figure 3.3A and B). Animals were exposed to the restrainer on training days pre-testing to acclimatise them to the device. After several days acclimatisation the animals did not exhibit obvious stress behaviours and seemed to tolerate restraint very well. They displayed sniffing and exploratory behaviour with their freely moving head and urine and faecal output were as normal. However the possibility that restraint may affect behaviours could not be ruled out. The Von Frey filaments were applied perpendicularly against the face on both sides and the threshold taken as the force at which the head was withdrawn or moved away from the stimulus. The lowest force to which the animal responded to three applications was scored as the value for that testing session.

An indication that cold allodynia was present in trigeminal nerve tested animals was given by their avoidance response to 0.1ml drop of acetone on the injured side of face. This did not occur with room temp water of with acetone on the contralateral side of face. However these responses were not recorded exhaustively and are not described in this thesis. The magnitude and composition of the reflex responses are also clearly different between the spinal and trigeminal models.

3.3.3 Statistical analysis

In each behavioural study, stimuli were applied to either paw several times and the data were averaged for each test day. (figures 3.1.and 3.3 inset graphs) Group averages are shown ± the standard error of the mean (SEM). For thermal behavioural tests (carried out only in the spinal model), to determine any significant differences between ipsilateral and contralateral hind limb values at specific time points post-surgery, a Student's matched pair t-test was performed on data. To determine differences between pre-and post- surgery values a repeated measures ANOVA followed by Dunnett's post hoc analysis was used. Both these tests are parametric tests as thermal data were continuous.

For data from von Frey filament testing (carried out in both the spinal and trigeminal models), to determine any significant differences between ipsilateral and contralateral hind limb values at specific time points post-surgery, a Wilcoxon test was performed. To determine changes in response scores over the test period (pre-

operative against post-operative) within each group, a Friedman test on ranks followed by Dunn's post hoc analysis was performed (also called a Dunn's method ANOVA). Due to the discrete and discontinuous nature of the mechanical data, i.e. von Frey filament forces being a graded series of forces in intervals, tests conducted on these data were non-parametric. Differences were considered statistically significant at $p \leq 0.05$.

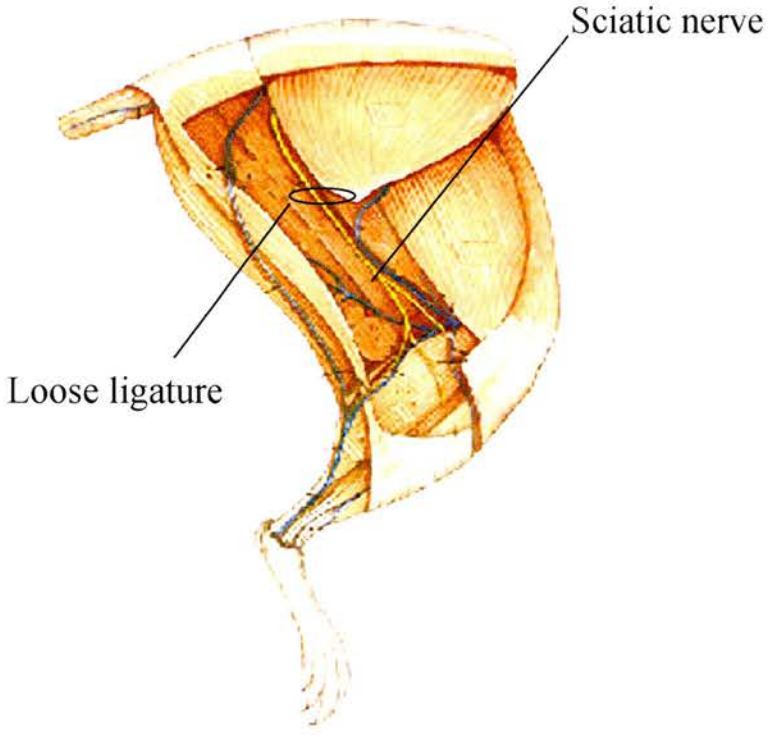
In each of the behavioural tests employed in either spinal or trigeminal preparations, there was no evidence for statistically significant contralateral changes compared to pre-surgery values, under the present experimental conditions.

Figure 3.1
Loose Ligation of Sciatic Nerve and Sensory Reflex Behaviours

A) Schematic diagram of rat leg dissected showing sciatic nerve and position of ligature placement in the chronic constriction injury. Four ligatures are loosely applied around the sciatic nerve at approximately 1mm intervals. B) Graph of mean results from behavioural testing of nine rats to von Frey filament application, which shows increased responsiveness of side ipsilateral to nerve injury (indicated by the symbol ††), from day 4 post surgery onwards. Statistical significance of differences between ipsilateral (○) and contralateral (■) paws post-surgery ($P<0.05$) was determined by a Mann-Whitney Rank Sum test. Significant difference of pre-surgery values to post-surgery values (indicated by ** symbol) was determined by a Kruskal-Wallis test with Dunn's post analysis ($P<0.05$). Each value is the mean ± SEM. There was no significant change in the contralateral side of the face.

Sciatic Chronic Constriction Injury and Sensory Reflex Behaviours

A)



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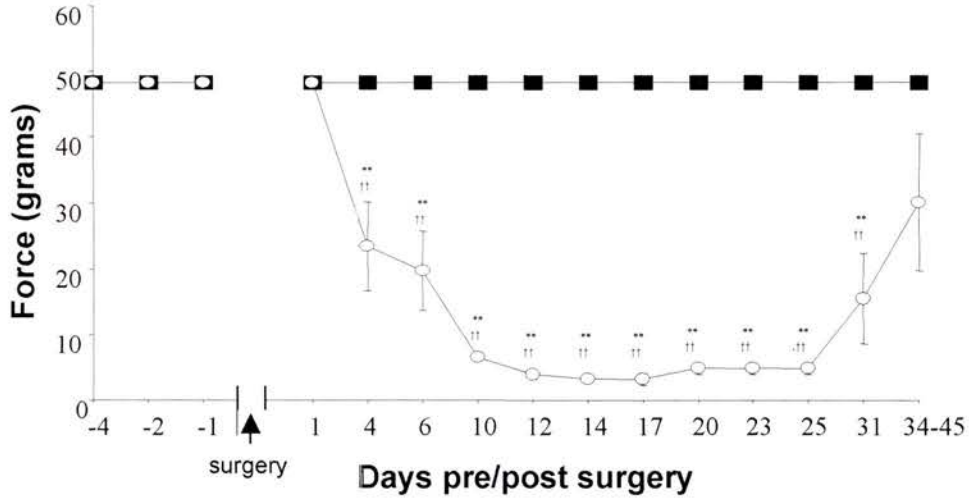
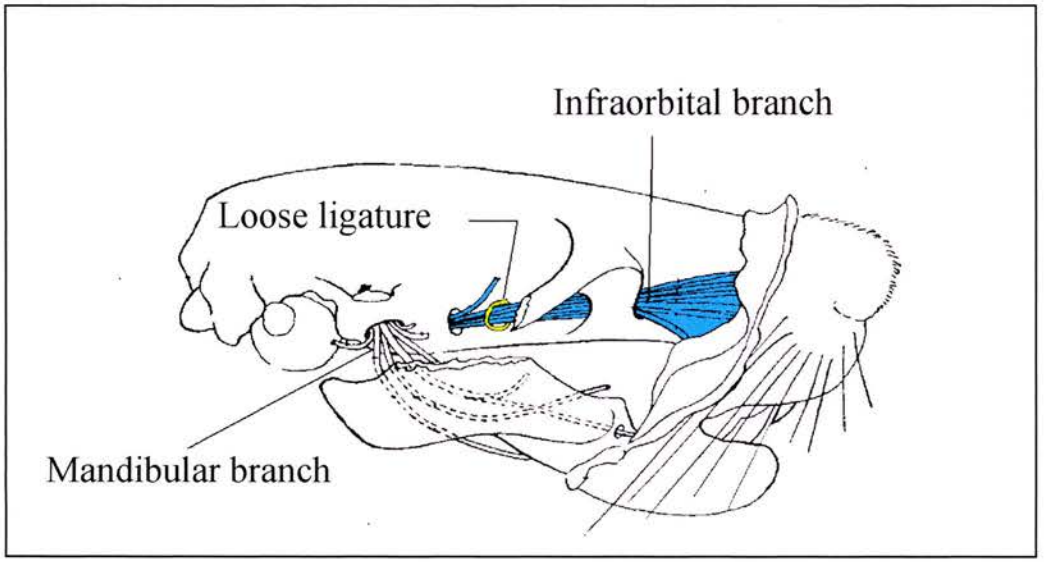


(adapted from Popesko et al.1990).

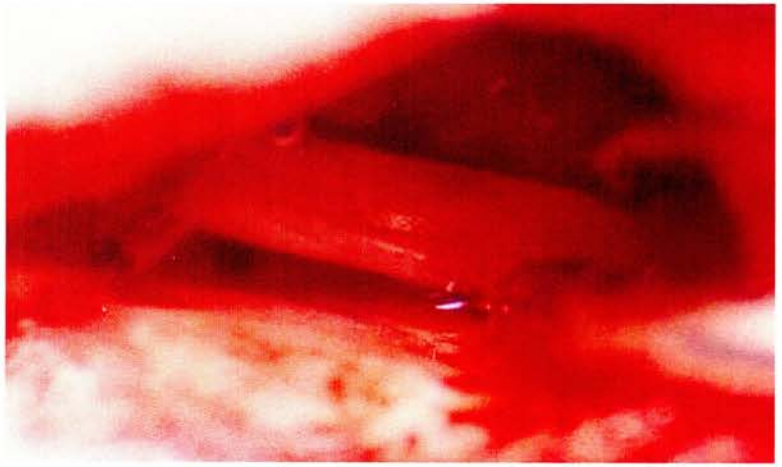
Figure 3.2
Loose Ligation of the Trigeminal Nerve

Inset schematic shows position of ligatures loosely applied around the infraorbital branch of the trigeminal nerve as it travels along the floor of the orbital cavity. Two ligatures are applied approximately 2mm apart. Picture A) shows a photograph taken of the dorsal view of the exposed nerve dissected free from surrounding connective tissue within the orbit and slightly raised on to two hooked retractors. Picture B) shows exposed nerve with ligatures in place.

Trigeminal nerve ligation



A



B

Chapter 4

The effect of null expression of α- synuclein or Persyn (γ-synuclein) on reflex pain behaviours following chronic constriction injury of the sciatic nerve

4.1 AIM

The aim of this study was to evaluate the consequences of null expression (i.e. the lack of expression) of either α-synuclein or persyn (γ-synuclein) on animals which had undergone nerve injury and compare them with wild-type littermates (that is, mutant and wild-type animals sharing the same background parentage) which have also undergone nerve injury. These proteins may be involved in the structural changes and synaptic plasticity known to be a key feature of nerve injury-induced pain states.

Some of the structural changes include

1. Trans-synaptic degeneration of inhibitory spinal neurons, which may result in both disinhibition and abnormal synaptic influences (Sugimoto et al, 1990; Ralston et al, 1997). This may be affected by α-synuclein which is strongly expressed in presynaptic terminals (Maroteaux and Scheller, 1991; Iwai et al, 1995), as it displays neurotoxic effects in vitro (El-Agnaf et al, 1998; Ostrerova et al, 1999), although it is not clear that synuclein plays any direct role in apoptotic cell death in vivo (Kholodilov et al, 1999). α synuclein over expression has been shown to inhibit PKC activity (osterova 1999) which may indicate in turn that α synuclein could possibly disrupt neurotransmitter vesicle regulation (Stevens and Sullivan, 1998). Also, α synuclein may simply exert a neurotoxic effect in the inhibitory interneuron cells themselves (El-Agnaf et al, 1998).

2. Regeneration of peripheral axons. As previously mentioned in the introductory section 1.10.1, the narrowing of axonal diameter (which occurs until connections are

re-established) is accompanied by a reduction in NF synthesis and levels (Hoffman et al 1985; Scott et al, 1999). Sciatic nerve injury causes marked alterations in the expression of afferent cell proteins, thought to be necessary for axonal regeneration, for example, reduced expression of NF proteins and increased tubulin and GAP-43 (Hoffman, 1989; Wong and Oblinger, 1990) expression. Cellular processes requiring the integrity of NF for their maintenance or cellular changes such as responses to injury, regeneration and growth, which may require turnover of neurofilament proteins, may well be influenced by Persyn (γ-synuclein) (Buchman et al 1998) and could also potentially influence the central synaptic plasticity that results from chronic constriction injury (CCI).

Evidence that synaptic changes may be influenced includes;

1. α-synuclein has been shown to modulate paired stimulus depression (i.e. two stimuli delivered in sequence and timed to cause a depression in synaptic responses) in hippocampal synapses (Abeliovich et al, 2000).
2. In mature hippocampal neurons, α-synuclein is localised almost exclusively to presynaptic terminals and downregulation of α-synuclein caused selective reduction in presynaptic vesicular pool size (Murphy et al, 2000), possibly affecting transmitter/modulator release.
3. One of the reported binding targets for α-synuclein is protein kinase C (Ostrerova et al, 1999). This is known to be important in neuropathic sensitisation in the spinal dorsal horn through interaction with receptors such as metabotropic glutamate receptors (Young et al, 1995).

This study was undertaken to evaluate the behavioural responses to both mechanical and thermal stimuli of α-synuclein null (non-expressing) mice following chronic constriction injury to the sciatic nerve compared to wild-type mice. The purpose of this evaluation is to examine whether the phenotypes that these mutant mice display with respect to sensory transmission warrant further investigation.

Methods

Most of the methods used in this chapter have been described in the general methods section of chapter 3. However the generation of synuclein knock-out mice is described below.

Generation and confirmation of synuclein knock-out mice

γ-synuclein mice were generated by the deletion of exons I, II, and III and the promoter region of the mouse γ-synuclein gene by homologous recombination. Confirmation of gene deletion and null expression of mice was by PCR-based genotyping of mice from a litter of two heterozygous parents, expression of mRNAs encoding members of the synuclein family in the retinas of wild-type and γ-synuclein null mutant mice and also by, results of Northern hybridization with a full-length mouse γ-synuclein cDNA probe, a mouse γ-synuclein-specific probe, a mouse γ-synuclein -specific probe, and a *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) probe. This is further described in detail in Ninkina et al, 2003.
Generation and confirmation of α-synuclein null mutant animals was as has been previously described (Abeliovich et al, 2000).

4.2 Results

General observations

Regular inspections were made for signs of autotomy, and notes were also made of the animal's gait, and the posture and condition of the affected hind limb. Both α-synuclein and persyn animals appeared slightly more restless than wild-type mice, with longer duration of exploratory activity and shorter periods of inactivity. The majority of nerve-injured animals showed the behavioural alterations characteristic of the CCI model. All of the animals which underwent surgery were protective of their affected hind paw, guarding it from incidental contact and rarely, if ever, extending the ipsilateral limb. The affected paw was generally held awkwardly with the toes tightly drawn together and ventroflexed. The animals

walked with a definite limp and a number of mice walked without allowing the affected paw to touch the floor at all. These abnormalities in posture are thought to be indicative of a chronic sensitised pain state (Attal et al, 1990).

With these exceptions, the animals' health and behaviour were generally normal and there were no apparent signs of debilitating pain or distress. The level of general activity appeared to be normal, and the animals could be handled without evoking squealing or biting. In addition, the animals appeared to gain weight in accordance with control animals. None of the animals exhibited any signs of autotomy.

Chronic constriction injury induces thermal hyperalgesia in α-synuclein and Persyn (γ-synuclein) null mice as well as wild-type mice.

Wild-type animals

In tests for thermal hyperalgesia, prior to the experimental nerve injury, wild-type animals displayed an average paw withdrawal latency of 11.12 ± 0.75 s for the left hind paw and 11.1 ± 0.58s for the right paw (n=12), with no significant difference between right and left hind limb values (Figure 4.1a). After CCI surgery, significant decreases in paw withdrawal latency (PWL) were seen for the right hind paw (ipsilateral to nerve injury compared to the contralateral paw, $p \leq 0.05$, Student's paired t-test. The difference at 18 days did not reach statistical significance. The mean PWL for the ipsilateral paw was 8.6 ± 1.5s at day 12, 9.2 ± 1.0s at day 14 and 7.95 ± 0.95s at day 21, whereas that for the contralateral paw was 13.0 ± 0.60s at day 12, 12.9 ± 0.48s at day 14 and 12.5 ± 0.66s at day 21. Ipsilateral, but not contralateral, PWL values through the period 12-21 days post-surgery were also significantly less than pre-surgery values ($p \leq 0.05$ repeated measures ANOVA followed by Student-Dunnett's post hoc analysis).This shows a consistent development of thermal hyperalgesia over a period of two to three weeks post-surgery in wild-type mice.

α-synuclein mutant animals

Baseline measurements of α-synuclein mutants showed paw withdrawal latencies of 8.92 ± 0.3s and 9.96 ± 0.6s for contralateral and ipsilateral paws respectively, with no significant difference between them (n=6; Fig 4.2a). Following surgery, however, significant differences were seen between paws at days 12-27, $p \leq 0.05$ Student's paired t-test. At corresponding times post-surgery the ipsilateral (but not contralateral) PWL values were significantly less than pre-surgery values ($p \leq 0.05$, repeated measures ANOVA followed by Dunnett's post hoc analysis). The greatest difference was at day 12 post-surgery with PWL values of 5.84 ± 0.7s for the ipsilateral paw and 11.2 ± 0.7s for contralateral paw. This development of behavioural sensitivity was similar to that in wild-type animals,

Persyn (γ-synuclein) mutant animals

Pre-surgery baseline measurements indicated paw withdrawal latencies of 9.31 ± 1.0s 8.89 ± 0.77s for contralateral and ipsilateral paws respectively (n=6), being very similar to their wild-type littermates. Post-surgery testing revealed significant differences between paws at days 12,-21, $p \leq 0.05$, Student's paired t-test. When compared to pre-surgery PWL values, the ipsilateral paw showed significantly reduced latencies at days 12-21; (repeated measures ANOVA followed by Dunnett's post hoc analysis, $p \leq 0.05$). The greatest difference was observed at day 18 post-surgery with PWL values of 6.8 ± 1.0s for the ipsilateral paw and 12.4 ± 0.7s for the contralateral paw. Again these results were similar to wild-type animals.

Chronic constriction injury induces mechanical allodynia in α- and γ-synuclein null mice as well as wild-type mice.

Wild-type animals

Prior to the experimental nerve injury, the wild-type animals displayed an average withdrawal threshold (PWT) to von Frey filaments, applied to the glabrous surface of the hindpaw of 3.6g (401mN/mm^2) in both the left and right hind paws. After surgery however, there was a significant decrease in the response threshold of the nerve-injured side, but not the contralateral side (Fig 4.1b). From as early as day 12 post-operatively (days 12-39), there was a significant reduction in the withdrawal threshold of the ipsilateral hind paw 2.5g ± 1.0g (246 ± 2.3 mN/mm^2 when compared with the contralateral hind limb value 3.6g (401mN/mm^2); (Wilcoxon test, $p \leq 0.05$). Similarly, at days 12-39, the ipsilateral, but not contralateral PWT values were significantly less than pre-surgery values ($p \leq 0.05$, Friedman test on ranks, followed by Dunn's post-hoc analysis). So, forces that the mice did not respond to before the nerve injury now clearly evoked pain-related withdrawal reflexes, indicative of mechanical allodynia. There were only slight differences between the threshold values of the contralateral hind limb in the nerve-injured animals compared to pre-surgery values, over the eight week test period, which were not significant.

α-synuclein mutant animals

Before animals underwent nerve injury, they displayed a baseline average paw withdrawal threshold (PWT) of 2.9g (320mN/mm^2) in both the left and right hind paws.

After CCI there was a very clear decrease in the response threshold of the nerve-injured (but not the contralateral) side (Fig 4.2b). From day 12 post-operatively (days 12-27) there was a significant reduction in the withdrawal threshold of the ipsilateral hind paw compared to the contralateral paw ($p \leq 0.05$, Wilcoxon test). At 12 days post-surgery the ipsilateral PWT was 1.5 ± 0.6g (243 ± 60 mN/mm^2) when compared with the contralateral hind limb value of 2.9g (320mN/mm^2). Ipsilateral, but not contralateral PWT values from days 12-27 were also significantly reduced

compared to pre-surgery values ($p \leq 0.05$, Friedman test on ranks, followed by Dunn's post-hoc analysis). Post-surgery reflex withdrawal behaviours between α-synuclein-null and wild-type animals appear very similar.

Persyn (γ-synuclein) mutant animals

Prior to the experimental nerve injury, these animals displayed an average paw withdrawal threshold (PWT) of 3.6g (401mN/mm^2) in both the left and right hind paws (Fig 4.3b).

After CCI, there was a clear decrease in the response threshold of the nerve-injured, but not contralateral side. There was a significant difference between ipsilateral and contralateral paws at days 12-43 post-surgery ($p \leq 0.05$, Wilcoxon test). The maximal difference was seen from days 12-21 where the mean PWT values remained at 1.4 ± 0.6g (247± 32 mN/mm^2) for the ipsilateral paw in comparison to 3.6g (401mN/mm^2) for the contralateral paw.

Similarly, the ipsilateral, but not contralateral PWT values (days 12-43) were significantly reduced from pre-surgery values ($p \leq 0.05$, Friedman test on ranks, followed by Dunn's post-hoc analysis). The profiles of post-surgery behaviours appeared very similar between persyn null animals and wild-type animals.

Figure 4.1
Behavioural Analysis of wild-type with Chronic Constriction Injury (CCI) to the Sciatic Nerve

(A and B) Data show mean responses for each day before and following the induction of CCI with error bar showing standard error of the mean (SEM). A variation of the chronic constriction injury (CCI) model for rat was used, whereby (under halothane anaesthesia) three ligatures separated by 1 mm were tied loosely to constrict the tibial branch of the sciatic nerve.

In wild-type mice (A), paw withdrawal latency (PWL) from a noxious thermal stimulus (Hargreaves' thermal stimulator) ipsilateral to CCI (open circle) showed significant differences between postoperative and preoperative values (cross indicates $p < 0.05$; Repeated measures ANOVA followed by Dunnett's post hoc analysis) and from postoperative, contralateral (closed squares) values (asterisk indicates $p < 0.05$ by Student's matched pair t test).

(B) Paw withdrawal thresholds (PWT) from mechanical stimulation (von Frey filaments) showed significant differences between postoperative and preoperative values on the side ipsilateral (open circle) to CCI (cross indicates Friedman test on ranks, followed by Dunn's post-hoc analysis $p < 0.05$) and between postoperative ipsilateral and contralateral values (asterisk indicates $p < 0.05$, Wilcoxon test). No significant differences were seen on the contralateral side (closed squares).

Figure 4.1

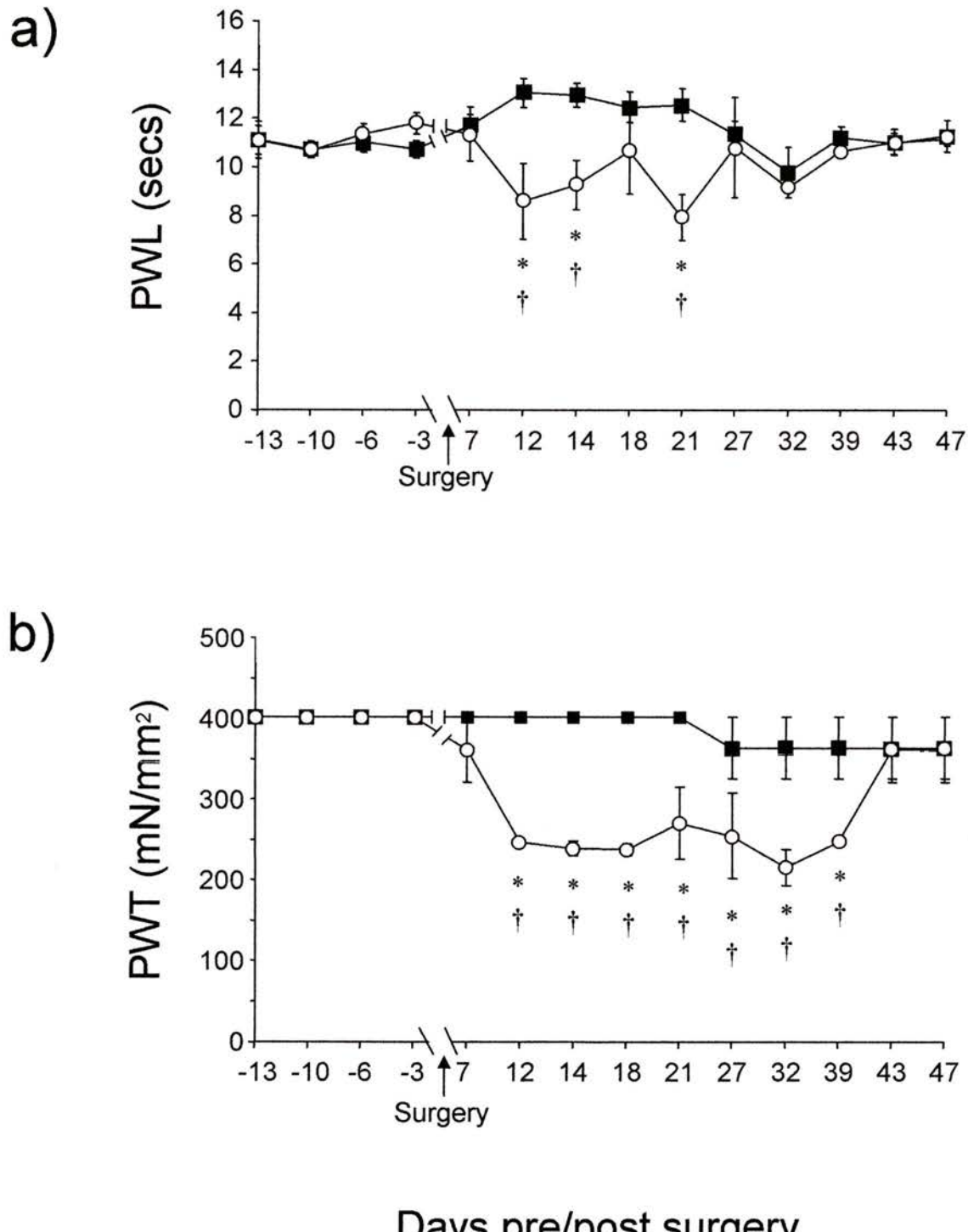

a)
PWL (secs)
16
14
12
10
8
6
4
2
0
-13 -10 -6 -3 7 12 14 18 21 27 32 39 43 47
Surgery
b)
PWT (mN/mm²)
500
400
300
200
100
0
-13 -10 -6 -3 7 12 14 18 21 27 32 39 43 47
Surgery
Days pre/post surgery

Figure 4.2
Behaviours of α-synuclein-null mice following CCI to the sciatic nerve

(C), paw withdrawal latency (PWL) from a noxious thermal stimulus (Hargreaves' thermal stimulator) ipsilateral to CCI (open circle) showed significant differences between postoperative and preoperative values (cross indicates $p < 0.05$; Repeated measures ANOVA followed by Dunnett's post hoc analysis) and from postoperative, contralateral (closed squares) values (asterisk indicates $p < 0.05$ by Student's matched pair t test).

(D) Paw withdrawal thresholds (PWT) from mechanical stimulation (von Frey filaments) for α-synuclein mice showed significant differences between postoperative and preoperative values on the side ipsilateral (open circle) to CCI (cross indicates Friedman test on ranks, followed by Dunn's post-hoc analysis $p < 0.05$) and between postoperative ipsilateral and contralateral values (asterisk indicates $p < 0.05$, Wilcoxon test). No significant differences were seen on the contralateral side (closed squares).

Figure 4.2

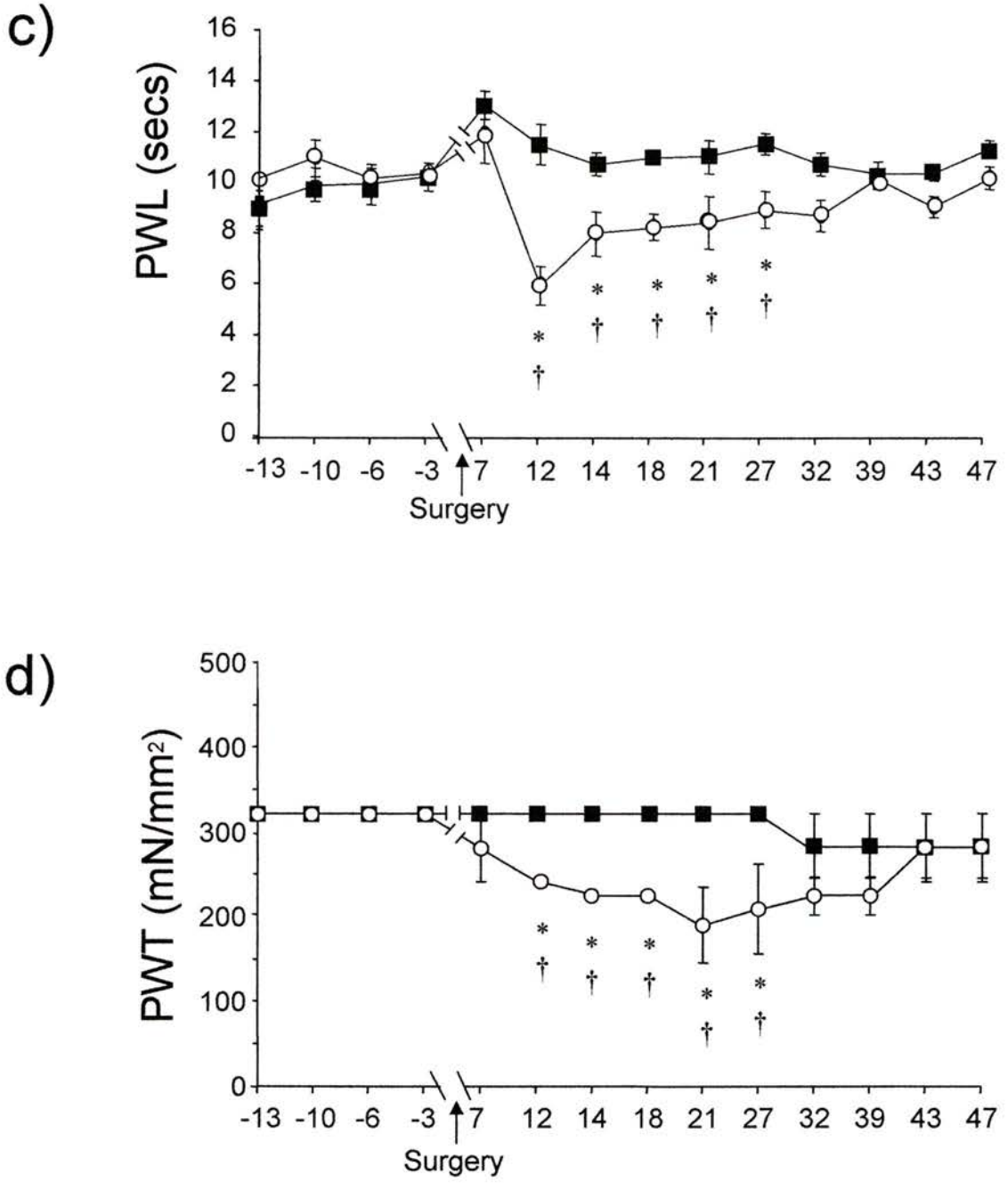

c)
PWL (secs)
16
14
12
10
8
6
4
2
0
-13 -10 -6 -3 7 12 14 18 21 27 32 39 43 47
Surgery
d)
PWT (mN/mm²)
500
400
300
200
100
0
-13 -10 -6 -3 7 12 14 18 21 27 32 39 43 47
Surgery
Days pre/post surgery

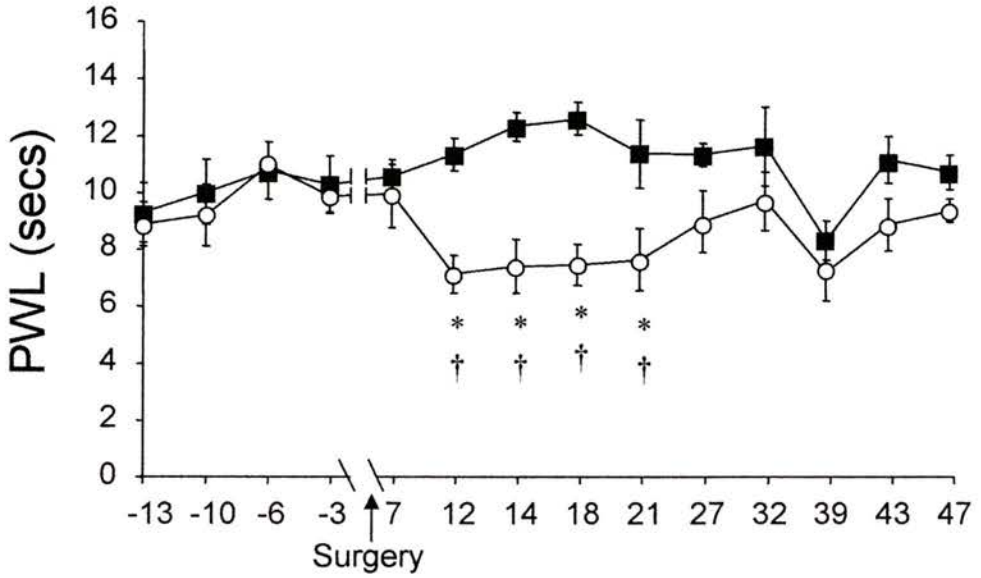
Figure 4.3
Behaviours of the Persyn-null mice following CCI to the sciatic nerve

(E), paw withdrawal latency (PWL) from a noxious thermal stimulus (Hargreaves' thermal stimulator) ipsilateral to CCI (open circle) showed significant differences between postoperative and preoperative values (cross indicates $p < 0.05$; Repeated measures ANOVA followed by Dunnett's post hoc analysis) and from postoperative, contralateral (closed squares) values (asterisk indicates $p < 0.05$ by Student's matched pair t test).

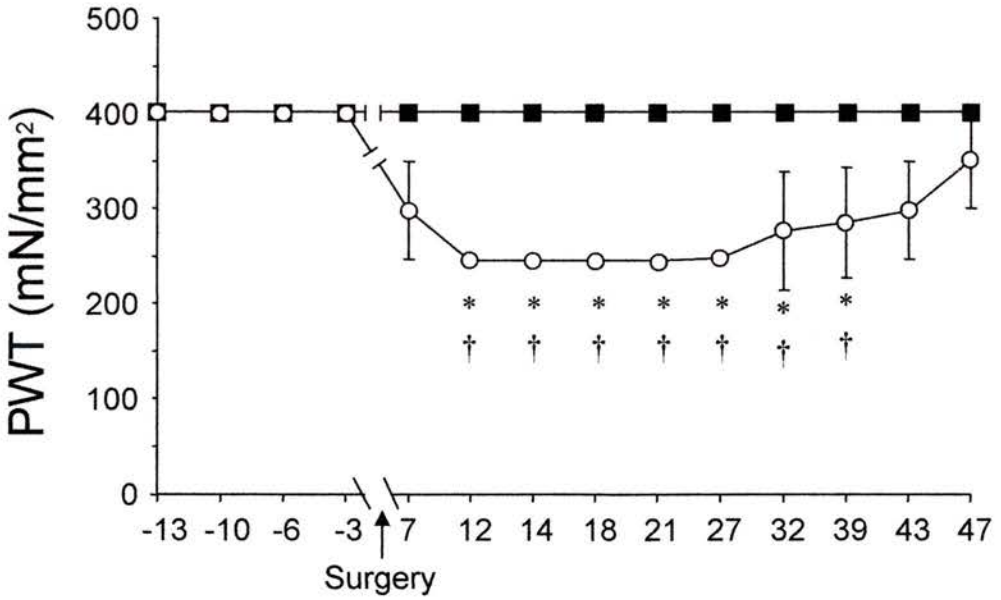
(F) Paw withdrawal thresholds (PWT) from mechanical stimulation (von Frey filaments) showed significant differences between postoperative and preoperative values on the side ipsilateral (open circle) to CCI (cross indicates Friedman test on ranks, followed by Dunn's post-hoc analysis $p < 0.05$) and between postoperative ipsilateral and contralateral values (asterisk indicates $p < 0.05$, Wilcoxon test). No significant differences were seen on the contralateral side (closed squares).

Figure 4.3

e)



f)



Days pre/post surgery

4.3 Discussion

The results for wild-type mice are broadly consistent with previous data showing the development of abnormal behavioural responses in animals which have undergone an experimental peripheral mononeuropathy (Attal et al. 1990; Bennett and Xie, 1988; Garry et al 2003). All animals following surgery showed an abnormal holding position of the affected hind paw. This behaviour is thought to be indicative of spontaneous pain (Attal et al. 1990). It is possible that this abnormality may be due to motor impairments following the nerve damage, but this seems unlikely as the rat holds the affected limb in particular protective positions rather than dragging the hind limb, as would be expected were it due to weaknesses in the dorsiflexor muscles of the hind limb. It seems more likely that the abnormal position of the paw predominantly reflects a reluctance of the rat to press its paw onto the ground, and that it is indeed a protective guarding behaviour.

If anything, the results from this group of wild-type mice are less profound than usually seen in similar experiments conducted within this laboratory. This variability in behavioural patterns may be due to slight differences in the "tightness" of the nerve ligatures, as it was difficult to ascertain a common "pressure" when tying the ligatures in individual animals, it is possible that variations in ligation tightness may lead to differences in the degree of degeneration within the nerve and subsequently differences in the development of the chronic pain conditions.

In these experiments, clearly the results for both α-synuclein and persyn animals are very similar to those of the wild-type littermates. Although some small differences do exist these are minor and do not constitute a deviation from the normal presentation of several groups of wild-type animals that is likely to have physiological implications.

In response to thermal stimulus tests, α-synuclein null animals marginally elongated thermal hyperalgesia, lasting until day 27 compared to day 21 post-operatively for wild-type animals (as also for γ-synuclein knockouts), but this is likely to be within the range of normal experimental variability. There was little difference in the amount of sensitisation displayed, however, between any of the groups. With regard

to expression of sensitisation to mechanical stimuli, again α– and γ-synuclein knockout mice developed ipsilateral allodynia similarly to wild-type animals.

A trend towards a lengthened duration of mechanical allodynia can be seen in the γ–synuclein mice, as well as a more somewhat marked shortening of the period of allodynia in the α–synuclein knockouts.

In conclusion, neither qualitative not substantial quantitative differences can be observed between α-synuclein or γ persyn mutant groups and wild-type littermates. Thus, these two proteins that are thought to be involved in cytoskeletal and synaptic events do not appear to have a major influence on the development, maintenance or recovery of neuropathic pain behaviours. We have been unable to show a role for α– and γ-synuclein in the development of neuropathic sensitisation of reflex pain behaviours. As a result of these findings it was deemed that these two proteins did not warrant further examination at this time.

Chapter 5

Evidence for differences in central sensitisation phenomena between the spinal cord and trigeminal system.

5.1 AIM

Extracellular recordings from single cells in the dorsal horn of the spinal cord have shown a linear increase in neuronal activity in response to graded mechanical stimulation (Werner and Mountcastle, 1965). However, although the phenomenon of "wind-up" the progressive, frequency-dependent facilitation of the responses of a neuron observed on the application of repetitive (usually electrical) stimuli of constant intensity has been shown in the spinal cord in response to electrical stimulation of afferent inputs (Mendell and Wall, 1965), and others have demonstrated a facilitated response of dorsal horn neurons to more "natural" types of stimuli, such as crushing of the hind paw (Sandkuhler and Liu, 1998; Dickinson et al, 1999; Palecek et al, 1992), as yet, the detailed electrophysiological investigation of the rate of change of response of neurons following nerve injury has not been fully examined. The unveiling of this change in responsiveness is not only further substantiating evidence for the existence of a central sensitisation following nerve injury, but would also be the first step in aiding the deciphering of the electrophysiological components of this state.

The aim of this study was to examine the sensory responses of neurons, in the dorsal horn of the spinal cord and in the trigeminal nucleus caudalis, to natural quantified mechanical and, in some cases cold stimuli, in both CCI and non-injured animals. Responses were divided into the "initial response" which was defined as the part of the response which occurred for the duration of the stimulus, and the "post-stimulus discharge responses (PSDR)" which was defined as any activity (above baseline values) that occurred as a result of the stimulus but occurred after the stimulus application. The PSDR was defined as: the response outlasting stimulus having

length >1200 ms, where length is computed by taking the time between response peak and the point where the response drops below two standard deviations above background activity (minimum 5 +/- 1 spikes per second, computed over 1s bins). The purpose of this was to observe and evaluate any differences in neuronal responsiveness that might occur between the naïve and neuropathic state. Also, it was our intention to compare and contrast any differences in neuronal sensory responsiveness that may occur in both the spinal cord and the trigeminal system. From collected data, stimulus response curves were constructed which allowed any difference in responsiveness or sensitisation to be detected as a shift in EC_{50} value (i.e. the magnitude of a stimulus which produces 50% of the maximum possible response for that stimulus) thus allowing comparison between groups.

Surgical Procedure for Preparation of Spinal Electrophysiological Recordings

Only nerve injured animals which showed strong signs of hyperalgesia and allodynia were used for electrophysiological recordings. Normal, unoperated rats were used for controls. Anaesthesia was induced with halothane to allow cannulation of the jugular vein, following induction, intravenous α-chloralose (60mg / kg) and urethane (1.2mg / kg) were given as required for maintenance of anaesthesia with supplementary doses of α-chloralose when required. Anaesthesia depth was assessed via observation of respiratory rate, and blood pressure was assessed by perfusion/colour of the iris of the eye. A tracheotomy was performed to maintain an unobstructed airway and the rat was allowed to breathe freely. Oxygen (0.1 L/ min) was passed over the end of the cannula to enrich the inspired air. Core temperature was maintained at 37-38°C by means of a thermostatically controlled heated blanket and rectal probe. (Figure 5.1) For spinal preparations the animal was mounted in a rigid stereotaxic frame and spinal segments L1-L6 were identified and the thoraco-lumbar spinal column was supported using 3 pairs of swan-necked clamps on alternate segments, with the middle clamp supporting L3. A dorsal laminectomy performed under x 12.5 magnification to expose segments L1-L6. To improve the stability of the preparations for extracellular recording, a well was made with skin flaps around the area of interest and agar solution (2% in 0.9% saline solution) at

39°C was injected under the most rostral and caudal vertebrae and then poured over the entire area and allowed to set. Once cooled, a narrow core of agar was removed to expose the area from which the recordings would be made, the dura was carefully cut and a pool of liquid paraffin (at 37°C) applied to the exposed area to prevent dehydration.

Surgical Procedure for Preparation of Trigeminal Electrophysiological Recordings

For trigeminal preparations anaesthesia was induced with halothane to allow cannulation of the jugular vein, following induction, intravenous α-chloralose (60mg/kg) and urethane (1.2mg/kg) were given as required for maintenance of anaesthesia with supplementary doses of α-chloralose when required. A tracheotomy was performed to maintain an unobstructed airway and the rat was allowed to breathe freely. Oxygen (0.1 L/min) was passed over the end of the cannula to enrich the inspired air. (Figure 5.2) The animals' head was then mounted in a rigid stereotaxic frame and the musculature of the neck and occipital portion of the skull was reflected to expose the dorsal surface of cervical vertebrae C1-C2. To further increase access the first cervical vertebra was removed and the angle of the head adjusted to a 20 degrees downward tilt from horizontal. This allowed direct visualisation of the dorsal surface of the brainstem and dura. To improve the stability of the preparations for extracellular recording, agar solution (2% in 0.9% saline solution) at 39°C was injected under the most rostral and caudal vertebrae and then poured over the entire pool, including the spinal cord and allowed to set. Once cooled, a core of agar was removed to expose the area from which the recordings would be made, the dura was carefully cut and a pool of liquid paraffin (at 37°C) applied to the exposed area to prevent dehydration. The trigeminal recordings were surprisingly stable for the duration of the testing protocol (about 1.5 hours). This was achieved solely with the use of agar placed around and immediately adjacent to the recording site. Core body temperature was maintained at 37-38°C by means of a thermostatically controlled heated blanket and rectal probe.

Extracellular Recording

Extracellular recordings were made from single dorsal horn neurons using the central barrel of a 7 barrelled glass microelectrode, filled with 4M NaCl, (pH 4.0-4.5) and with tip size 4.0-5.5um. Side barrels contained either 1M NaCl (pH 4.5) or 2% Pontamine Sky Blue in 0.5M sodium acetate for histological marking of recording sites. Recordings were made at depths of 100-1000μm from the spinal cord surface (100-600μm in the trigeminal complex), and cellular activity was monitored via an oscilloscope (Tektronix). The oscilloscope was also used to aid neuron discrimination. The signal then passed to a D130 digitimer spike processor, with the spike discriminator adjusted to isolate one neuron at a time. Further, the signal was fed to a standard 1401 interface (CED systems, Cambridge) and neuronal activity was continuously plotted on-line using Spike 2 version 4.1 software (CED systems, Cambridge) on a RM PIII 500 PC workstation (IBM type).

Identification of Neuronal Receptive Fields

Neuronal receptive fields were identified by manually brushing the ipsilateral hind limb or side of the face, whilst lowering the electrode into the preparation using a microdrive. Neurons were further qualitatively examined using noxious pinch, cold (acetone) and, in spinal preparations only, heat (48°C for 10s). A cold stimulus was delivered using ~~with~~ a single drop (approximately 0.1ml) of acetone to the side of the face. The heat stimulus was applied using a ramp driven Peltier device (Medical Instruments) with a contact area of $1cm^2$. The Peltier probe, while in contact with the animals' skin, was raised from a resting temperature of 32°C to 48°C (ramp rate 5°C/s) to be held at 48°C for 10s. Quantitative data were obtained in both spinal and trigeminal preparations measuring responses to von Frey filament stimulation. In occasional examples in the trigeminal preparation, electrophysiological responses to unquantified cold stimuli were also monitored. The neurons were then classified according to their sensory input and those neurons classified as

being multireceptive (for example, those that responded to noxious and innocuous brush stimuli) were used for this study. Due to the search procedure protocol extracellular recordings were made from single, multireceptive neurons (LIII-V) in the dorsal horn and wide dynamic range neurons in the zonas magnocellualris in the caudalis, ipsilateral to the nerve injury, in neuropathic animals and bilaterally in unoperated control rats. It was hoped that recordings of neuronal responses contralateral to injury could have been made, but the length of time for ipsilateral recordings precluded any study of contralateral neuronal responsiveness'

Test stimuli were applied for approximately 1 second and as close as possible to the centre of the identified receptive field.

Analysis of Electrophysiological Data

Data were digitised and processed by computer to give a record of cell firing rate against time (spikes/s). Each application of the von Frey stimulus was for approximately one second and the time at which this application was applied was marked by a second operator on the computer trace. Each application was considered a "trial" and similar trials for each stimulus were averaged for each cell. Whilst analysing data from trials there was no subtraction of 'background' activity which was generally low at 0-5 spikes per second. As each trial that resulted in a response was collected, it could be quickly concluded that each response was not identical, some responses were short (lasting for the duration that the stimulus was applied and mostly from normal animals) whilst others were longer (lasting far in excess of the duration for which the stimulus was applied to the skin and mostly from neuropathic animals). Therefore, the data were analysed in several ways. In the first instance, short responses were analysed in terms of peak response. Secondly, prolonged responses that continued beyond the cessation of the stimulus, were divided into an initial part (the part of the response that corresponded to the duration of the stimulus applied) and a second part of the response which outlasted the stimulus and was termed the post stimulus discharge response (PSDR). The criterion for considering a response an after-discharge included any response with activity >2 standard

deviations above background (as estimated from spontaneous activity at the beginning of recording) in the interval of 1.5 to 2.5 seconds after the peak of the response. This interval was chosen to guarantee that the stimulus was no longer applied at this time.

From the short responses and the initial part of longer responses (falling within the time of stimulus application), stimulus response curves were constructed, taken from averaged values of trials from a number of cells from both normal and neuropathic animals. After-discharges were examined for duration, peak response, and total activity, both on their own and in combination with the initial part of the response. Other analysis included comparison of inter-spike intervals and the examination of spike timing in relation to the stimulus. Furthermore, the effect of or applying acetone to the receptive field prior to a stimulus was investigated. Previous to these experiments the threshold for mechanical stimuli was determined (the threshold force of a neuron is defined as the lowest von Frey force at which an after-discharging trial was observed for that neuron, not including trials after application of heat, cold, or brush stimulation). Threshold and twice threshold responses were then compared both before and after additional cold or brush stimuli were included in the testing regime.

Identification of recording sites

At the end of some experiments one barrel of the recording electrode was filled with Pontamine sky blue dye so that the recording site could be identified. After recording from a neuron, this dye is injected into the tissue at the point of recording using a 5 μA current for 10-15 minutes. When all recording sites were marked the tissue was removed and fixed in 4% paraformaldehyde for two hours. The tissue was then sectioned for viewing under a light microscope. Sections were not counterstained but were viewed under polarised light (fig 5.3). All areas of dye penetration are located and cross referenced with the recorded data.

5.2 Collection of electrophysiological data

Recordings in animals treated surgically were made when they showed peak neuropathic behavioural responses. This was usually between 12-16 days in spinal sciatic nerve CCI animals and 30-40 days in the trigeminal infraorbital nerve CCIio animals. Each application of a mechanical stimulus was termed a trial and multiple trials, usually three, were run on each neuron for each force. For graphs of initial response against force for each neuron, statistical analysis between points (i.e. the mean of responses collected from a number of animals at each force), were compared by Student's t-test.

When comparing PSDR trials from neurons from neuropathic animals in spinal cord and trigeminal system, the peak of a response was computed by convolving the response spikes with 25ms standard deviation Gaussians and taking the maximum of this spike density function. Threshold was defined as: the lowest force at which a neuron discharges. Initial spike counts were taken as the number of spikes in the interval of 500ms before to 700ms after response peak over the duration of the stimulus. Total spike count equals the number of spikes in the entire response and discharge rate is defined as, the average firing rate spikes per second in the interval of 1200ms after peak to end. Error-bars show the standard error of the mean.

Statistical analysis

A total of 55 neurons from 27 rats were examined, 22 from spinal cord recordings (8 neurons from normal rats and 14 neurons from neuropathic rats) and 33 from trigeminal recordings (7 neurons from normal rats and 26 neurons from neuropathic rats). In the spinal cord, the vast majority of neurons were within laminae III-V of the dorsal horn, as determined by histological marking with PSB, and corresponding to microdrive electrode depth readings of 0-1000μm from the spinal cord surface. These microdrive readings have also been shown to correlate well with the depth of the electrode tip in the spinal cord in a number of other electrophysiological studies within our laboratory (Munro et al. 1993; Young et al. 1994; 1995b; 1997). In the trigeminal caudalis, neurons were predominantly from the zonas magnocellularis as

determined by histological marking with PSB, at depths of approximately 0-900μm, recording sites are represented in figure 5.3. All neuropathic animals investigated were at the peak of neuropathic behavioural changes as assessed on the day of the recording experiment.

Background activity ranged from 0-2 spikes per second ± 0.3 in most neurons from naive animals and 4-7 spikes per second ± 2.3 for neurons from neuropathic animals, these background activities were the same for both trigeminal and spinal neurons.

5.3 Results

All neuropathic animals investigated in electrophysiological studies were tested behaviourally beforehand to ensure behavioural changes had occurred. Behavioural indices were measured in response to mechanical stimuli in both spinal and trigeminal models using reflex withdrawal tests to graded von Frey filaments (as described in section 3.3).

5.3.1 Chronic constriction injury causes a behavioural sensitivity in both spinal sciatic and trigeminal nerve injury models.

A repeatable and robust behavioural sensitivity to mechanical stimuli can be caused by chronic constriction injury to the nerve in both spinal sciatic and trigeminal nerve models. Reflex withdrawal tests show that following chronic constriction injury to the sciatic nerve of the hind limb, the force of mechanical stimulus needed to elicit withdrawal reflexes changed from the normal value of approximately 50 grams seen in naïve animals to a minimum of between 5-10 grams on the ipsilateral side from as early as 4-6 days post-operatively, whilst no significant contralateral change was observed.

Peak behavioural changes in spinal neuropathic animals were observed 7-14 post-operatively.

In response to chronic constriction injury to the trigeminal nerve there was a significant decrease in force of mechanical stimulus needed to elicit a behavioural reflex withdrawal on the injured side. This decreases from the normal range in naïve

trigeminal animals of between 8-12 grams on both the ipsilateral and contralateral side of the face, to between 3-5 grams ipsilaterally (n=9 as shown in fig 3.3 chapter 3), on average 25-30 days after injury, whilst no significant contralateral change was observed.

5.3.2 Neurons in the spinal dorsal horn and trigeminal caudalis of naive animals show intensity coding of graded mechanical stimuli.

Single cell electrophysiological recordings show that neurons recorded in the spinal dorsal horn in normal animals exhibited a linear increase in neuronal activity as the force of mechanical stimulus increased (figure 5.4). In naïve animals this activity ranged from approximately 2-3 spikes per second at forces of <1 gram, to 10-15 spikes per second at forces around 15 grams. This approximately linear intensity coding of mechanical stimuli is markedly facilitated in neuropathic animals, where activity ranged from 6 spikes per second at forces <1 gram to around 30 spikes per second at forces as small as 5 grams. In naïve trigeminal animals the intensity coding of mechanical forces is much steeper than that seen in naïve spinal cord neurons. The range of activity for neurons measured ranged from 5 spikes per second for a force of 0.6 gram, peaking at 40 spikes per second at a force of 4grams and falling to around 30-35 spikes per second for forces above 6grams.

This indicates that in neuropathic animals not only does activity reach levels at low forces only normally seen at higher forces in the naïve animals but also, activity can reach levels much higher than that ever seen in naïve animals at any of these forces.

5.3.3 Spinal and trigeminal neurons show marked differences in mean initial responsiveness between normal and neuropathic animals.

From all recordings of neurons in the dorsal horn of the spinal cord (8 neurons, 212 trials) of mechanical stimuli were conducted in normal animals (7 neurons, 354 trials) in neuropathic animals. Taking the initial part of the response and averaging all trials at each force the neuronal activity for neurons from naive animals increases

approximately linearly with force. However neurons from spinal neuropathic animals show a markedly greater rate of neuronal activity (figure 5.4). This change in rate is shown by a significant difference between groups at all points above 1 gram in force. Initial responses in spinal neurones were typically 2 spikes per second for naïve and 12 spikes per second for neuropathic at 0.4 g forces, with corresponding responses at forces above 9 g of around 7 spikes per second and 20 spikes per second respectively Figure 5.5 shows that whereas spinal neurons exhibit a clear increase in the rate of activity between naive and neuropathic states, trigeminal neurons show minimal differences as well as an already high rate of activity in the trigeminal naïve neurons. Figure 5.4 shows individual responses of spinal and trigeminal neurons from naïve and neuropathic animals. Spinal cord dorsal horn neurons in naïve animals (figure 5.4a) show responses of around 4 spikes per second at 1 gram and 15 spikes per second at 9 grams, neuropathic animals however show much facilitated responses with 5 spikes per second at 0.4 gram 25 spikes per second at 1 gram and 60 spikes per second at 9 grams.

In trigeminal animals the initial responses were facilitated to a lesser extent in neuropathic recordings with responses ranging from around 10 spikes per second at 0.4 gram, 40 spikes per second at 1 gram to 60 spikes per second at forces above 9 grams (figure 5.4d), compared to neurons in naïve animals which showed responses of 5 spikes per second at 1 gram and 14 spikes per second at 9 grams (figure 5.4b). Neurons in naïve animals did not show a response at 0.4 gram.

In naïve animals reflex withdrawal behaviours were caused by lower forces in the trigeminal neurons than in spinal neurons would be consistent with a greater responsiveness to mechanical stimuli in the trigeminal system compared to the spinal system in naïve animals, although direct comparisons of a quantitative nature should not be made without great caution because the two models are not identical in format.

5.3.4 Neurons in neuropathic animals show marked PSDR properties compared to neurons in naïve animals.

In total, from both the spinal cord and trigeminal caudalis, 55 neurons from 27 rats were tested (15 from normal rats and 40 from neuropathic rats). 17 of the 40 neurons from the neuropathic group showed PSDR properties whereas 2 of the 15 neurons from naive animals showed PSDRs. Fisher's exact test showed a p value of >0.1, but >0.5, so the proportion of neurons showing PSDR in neuropathic rats was not significantly greater than that in naives given the present relatively small group sizes. Further experiments to increase the group sizes may lead to the difference reaching statistical significance. Figure 5.4 (e and f) show typical examples of spinal and trigeminal neurones exhibiting PSDR properties, with the trigeminal neurone demonstrating a longer duration of afterdischarge (PSDR). These results were typical for the neurones that showed afterdischarge in both spinal cord and trigeminal nucleus through a range of stimulus intensities. Some afterdischarging trigeminal neurones showed briefer periods of afterdischarge, generally at lower stimulus intensities, but the prolonged afterdischarge seen frequently in trigeminal neurons and illustrated here was never observed in spinal neurones at any stimulus intensity tested.

5.3.5 Trigeminal neurons from neuropathic animals show much greater PSDR activity than spinal neurons from neuropathic animals.

Of 40 neurons from neuropathic animals 14 were from spinal cord and 26 were from the trigeminal caudalis. The PSDRs from trigeminal neurons elicited from a range of stimulus intensities (threshold and twice threshold) of between 2 and 8 grams were on average much longer than those seen in the spinal cord. The length of discharge was an average of 8333 ± 1610 action potentials from spinal cord neurons and 46390 ± 16026 action potentials for trigeminal neurons. This five fold difference in activity is confined to the length of discharge, as the rate of discharge is similar between groups. Strikingly, the average force at which PSDRs occur in the trigeminal system is approximately half of that in the spinal cord, with an average of 6.42 ± 0.49 grams

causing discharging activity in the trigeminal neurons and a average force of 13.64 ± 1.81 grams in the spinal cord (from stimulus intensities of between 8 and 15 grams) i.e. mean threshold force for spinal neurons was 2.1 fold that for trigeminal neurons. Caution is needed however in the interpretation of this quantitative difference because the sciatic and trigeminal CCI models are not identical in format.

5.3.6 Total response duration of trigeminal PSDR neurons shows marked difference from those of spinal PSDR neurons.

Figure 5.4 shows the total response of PSDR neurons to application of mechanical stimuli at a force approximately equal to half the force needed to elicit a PSDR response from that neuron, the threshold force needed to elicit a PSDR response, and twice the threshold force needed to elicit a PSDR response. Precise 2-fold ratios to the threshold force were difficult to achieve in practise because of neuron:neuron variation and the limited range of focus available in the von Frey filament set. In spinal cord neurons these forces were 4.8 ± 1.1g half threshold, 12.9 ± 2g threshold, and 17.9 ± 4g twice threshold. In trigeminal neurons they were 4.0 ± 0.4g half threshold, 8.4 ± 0.8g threshold, and 10.5 ± 0.8g twice threshold (these forces are not even because individual neurons reached threshold at different forces and therefore each value is a mean value. Also they are not multiples of two because the von Frey sequence is not linear and for example if threshold for one neuron is 2g then twice threshold would have been 4gs, but if threshold was 6g the nearest force of filaments to twice this would be 10g or 15g). The total responses at each force included the initial response, occurring over the duration of the stimulus and the full length of the PSDR. The total duration of this response was plotted against force. As can be seen in Figure 5.6, spinal neurons (n=5) exhibit a steady increase in the duration of the total response to increasing forces (with a mean total duration of 0.39 ± 0.07s at about half threshold, 9.30 ± 3.53s at threshold and 22.12 ± 7.15s at about twice threshold). The duration of responses at twice threshold was significantly greater than those at threshold (Mann-Whitney U test, $p<0.05$). However, trigeminal neurons (n=12), show an increase in the duration of their responses between half threshold

and threshold, but a marked and statistically significant decrease between threshold and about twice threshold, (with total mean durations of 0.52 ± 0.04s about half threshold, 9.73 ± 4.08s threshold, and 1.06 ± 0. 20s about twice threshold; Mann-Whitney U test, $p<0.05$). For trigeminal neurons at about twice threshold, the duration of responses is significantly less than it is at threshold, whereas for spinal neurons, the duration is significantly more than it is at threshold. All neurons used in this data had stopped firing after 45 seconds. The transition in responsiveness from threshold to high intensity mechanical stimuli is quite different between spinal neurons, where there is an intensity-related response and trigeminal neurons, where there is a lower threshold, but a reduction in responsiveness at higher intensities. The basis for this is unclear, but it is possible that high intensity inputs may invoke the activation of profoundly inhibitory central mechanisms and thereby actually suppress responsiveness to increasingly intense stimuli.

5.3.7 Neuropathic recordings can be subdivided to show two different responses of neurons.

When the neuronal responses to a range of von Frey filaments from neuropathic animals were further analysed they could be separated into neurons which showed PSDR properties and neurons that did not (figure 5.7). Although the total number of neurons recorded so far is relatively small, their responses appeared to fall into two distinct categories. Further replication of these data would be required to draw definitive conclusions about the two proposed subpopulations.

It can be seen by examination of initial responses, (averages taken over the first 1.2 seconds) in recordings from spinal preparations, that neurons from neuropathic animals that do not display after discharging activity (n=9) have a similar response to neurons from naïve animals (n=8). Indeed in most cases the mean values of these two groups are not significantly different (only means at forces 0.6g, 2g, 8g and 10g show significant from normal). However, neurons from neuropathic animals that do show PSDR activity (n=5) have very much higher levels of activity than that seen in neurons from naïve animals (means at all forces except 1g being significantly different from normal).

As can be seen in figs 5.4 and 5.7 neurons from the trigeminal caudalis of naïve animals showed generally higher levels of neuronal activity than those in spinal cord, to equivalent von Frey filaments. Although this difference was of interest, it is not appropriate to make direct quantitative differences between the two models because of potential differences in the influence of the lesion and in relative coverage of receptive fields in the two models. Furthermore, non-PSDR neurons from neuropathic animals show higher levels of activity in trigeminal neurons than spinal cord neurons even though non-PSDR trigeminal neurons show lower activity than trigeminal neurons from naïve animals.

5.3.8 Trigeminal neurons in neuropathic but not naïve animals show altered mechanical responses following acute brushing or cold conditioning stimuli to the face.

Trigeminal neurons were also tested for responses to von Frey filaments both before and after a brief brush or cold stimulus applied to the face, ipsilateral to injury. This protocol included either a 5 second brush of the receptive field and surrounding area, or placement of 1 drop of acetone to the receptive field and surrounding area, then a period of 2 minutes before subsequent re-application of the same von Frey filaments. For neurons tested in this manner, responses were again separated into both the initial response and PSDR for further analysis (figure 5.8). Using this protocol for before and after brush stimulation, 14 neurons from trigeminal neuropathic animals were tested. Of these, 7 neurons showed an increased initial response to low (4g) and high (15g) forces after the brush stimulus compared to that before the brush stimulus (4g force: 12.2 ± 0.9 spikes per second before and 24.3 ± 2.9 spikes per second after, n=7, p=0.038; Matched pair Student's t-test; 15g force: 19.6 ± 5.8 spikes per second before and 26.8 ± 7.0 spikes per second after, n=7, p=0.005; Matched pair Student's t-test). 7 neurons in normal animals were tested but no significant change in initial responses could be detected (4g force, 12.2 ± 2.1 spikes per second before and 11.2 ± 2.2 spikes per second after).

With respect to PSDRs, of the 14 neurons tested 2 were removed due to inconsistent responses leaving 12 neurons. Of these 12 neurons, 9 showed a sustained discharge that outlasted the stimulus period to stimulation above a threshold level. Of these 9 neurons, 6 exhibited longer PSDRs at low a force after brushing the face than before (4.3 action potentials ± 1.6 before and 80.8 action potentials ± 42.0 after at 4g $p=0.047$), but none at higher forces (10.7 action potentials ± 3.6 before and 10.5 action potentials ± 7.0 after, at 15g). 7 neurons in normal animals were tested but no significant change in PSDR responses could be detected.

Of the 9 trigeminal neurons above that showed PSDR activity, 6 also showed PSDR after a period of latency (average latency of 8.3s ± 3.3s). All PSDRs with latency follow after brushing stimulus to the face.

Using this protocol with cold, no change in the initial responses could been observed for either naive or neuropathic animals, however, in neuropathic animals exhibiting PSDR activity, 3 out of 12 neurons showed a significant decrease in PSDR after cold after cold stimulation of the face using acetone (18 action potentials ± 2.5 before and 0.66 action potentials ± 0.66 after). Each conditioning cold stimulus application elicited a response from the neuron being recorded but these responses were not analysed.

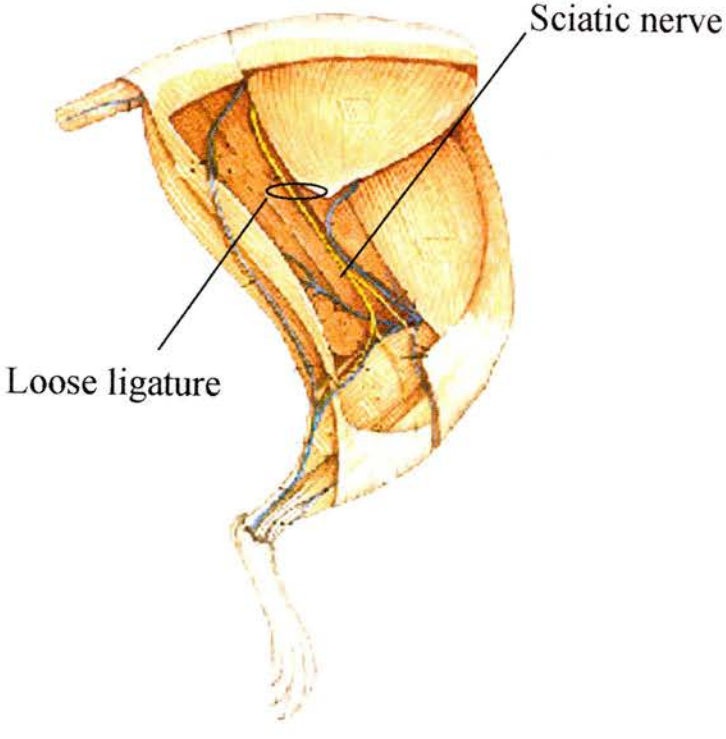
These conditioning stimuli experiments were not carried out in spinal cord preparation animals due to time constraints.

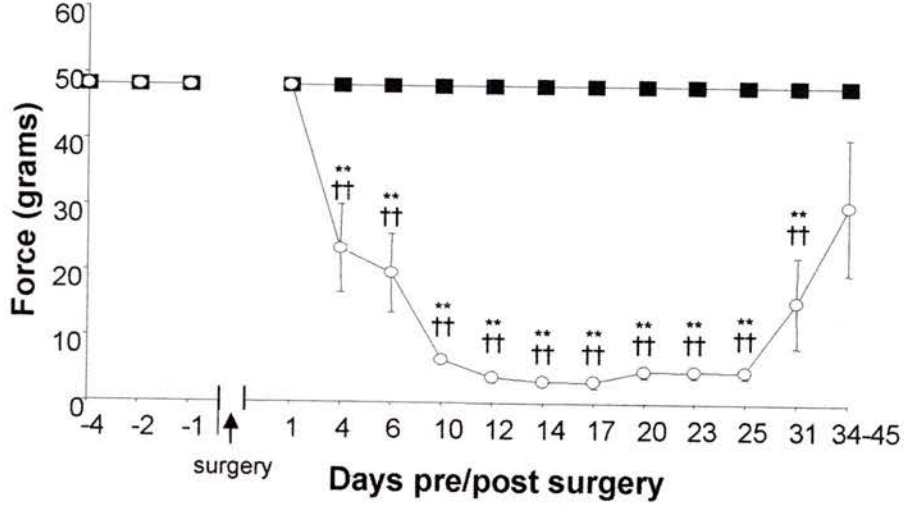
Figure 5.1
Spinal Electrophysiological Recording Experiments

Schematic representation of the recording set up for spinal electrophysiology experiments. The spinal cord vertebrae were stabilised using 3 pairs of swan necked clamps and a laminectomy performed to reveal lumbar segments L1-L6. Skin flaps were used to produce a pool around the area of interest, and this pool was filled with a 2% agar solution to provide stability during the recording. Following the removal of the agar core above the recording area, the dura was removed and the exposed spinal cord covered in 37C paraffin oil.

Extracellular recordings were made via a 7-barelled glass micro-electrode, and the recording signal amplified 1000 times before being transmitted to a spike processor where the number of action potentials per second were counted. Neuronal firing rate was continuously plotted on-line on an IBM computer. Neuronal activity was monitored on the oscilloscope screen.

Sciatic Chronic Constriction Injury and Sensory Reflex Behaviours





(adapted from Popesko et al.1990).

Figure 5.2
Trigeminal Electrophysiological Recording Experiments

Schematic representation of the recording set up for spinal electrophysiology experiments. The head was stabilised in a sterotaxic head frame and C1 removed for direct access to brainstem. An agar pool was made similar to that in figure 3.4 and all other recording was identical to that previously described.

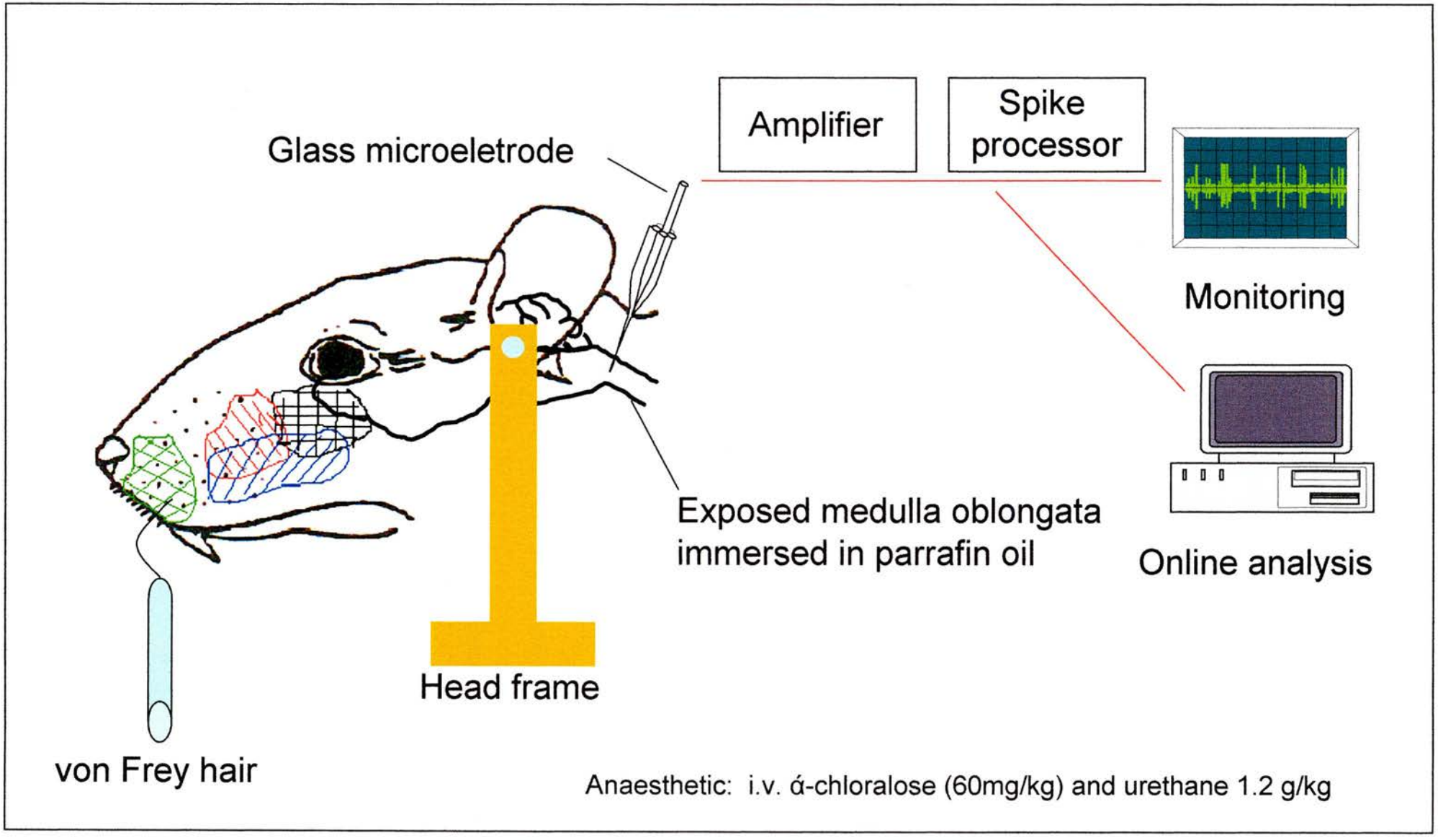

Amplifier
Spike processor
Glass microeletrode
Monitoring
Exposed medulla oblongata immersed in parrafin oil
Online analysis
Head frame
von Frey hair
Anaesthetic: i.v. ά-chloralose (60mg/kg) and urethane 1.2 g/kg

Figure 5.3

Staining of recording sites in trigeminal caudalis.

a) Photomicrograph showing blue staining of dye injected into recording site tract by ionophoresis, indicated by arrow. b) Illustration showing example locations of recording sites in the subnucleus caudalis. In this section lowest extent of tract marked as asterix, other example recording sites from different sections shown as circles. In all cases neurons recorded were from depths within which dye locations were obtained. Abbreviations (SP5C, trigeminal caudalis. CC, central canal; LRTN, lateral reticular nucleus; pyx, pyrimidal tracts.)

a)

b)

Figure 5.4

Typical examples of electrophysiological recordings of single spinal dorsal horn or trigeminal caudalis neurons to show the relative responses to single von Frey filaments of increasing force applied to their receptive fields on the face in naïve or neuropathic animals.

Each von Frey filament application duration was 1 sec (x2 applications represented by bar). (a) and c) show responses of spinal dorsal horn neurons in naïve and neuropathic animals respectively. Responses are typically seen at lower forces in neuropathic animals. (b) and d) show responses for trigeminal caudalis neurons, with same characteristic increase in responsiveness to lower forces shown in neuropathic animals.

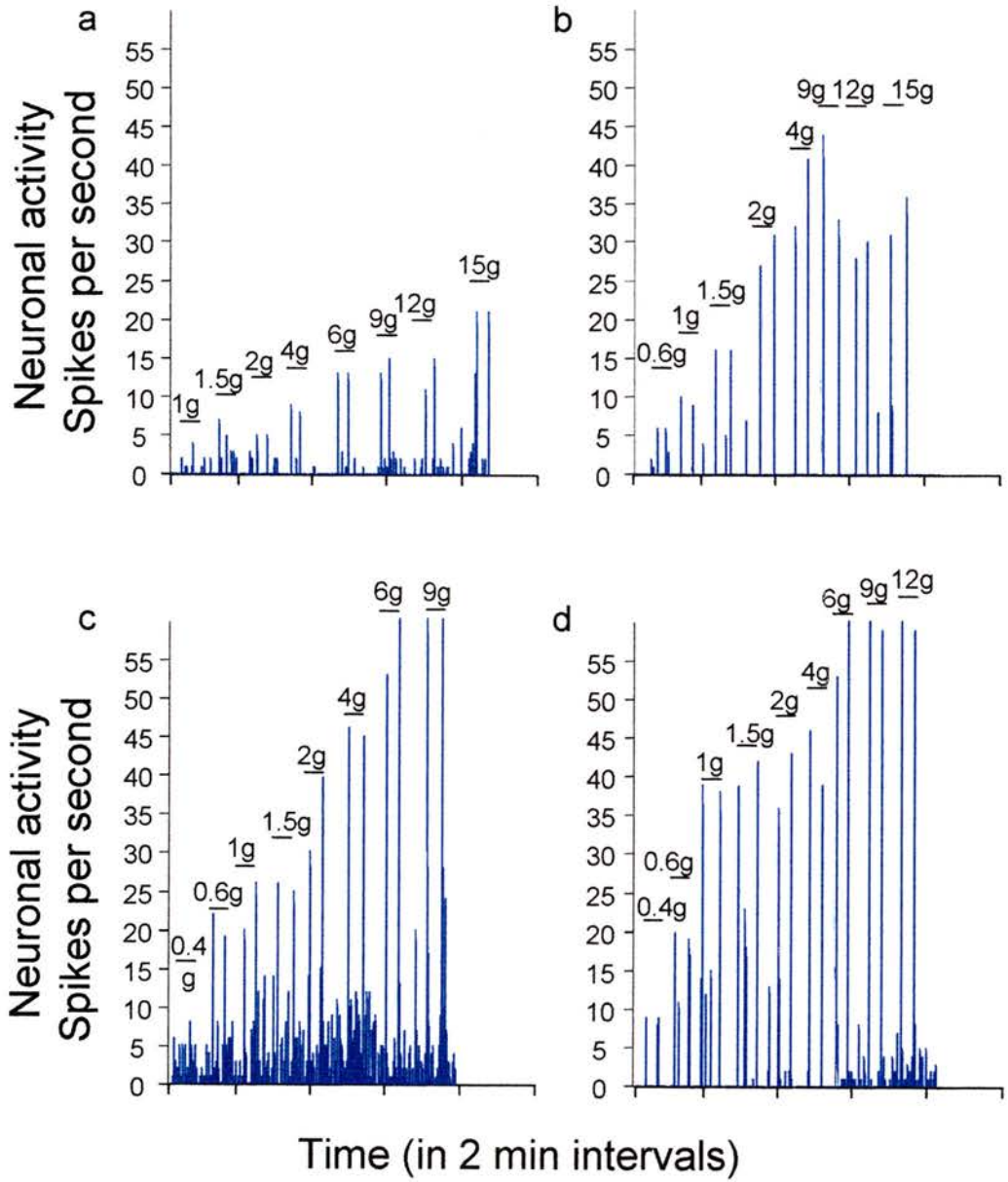
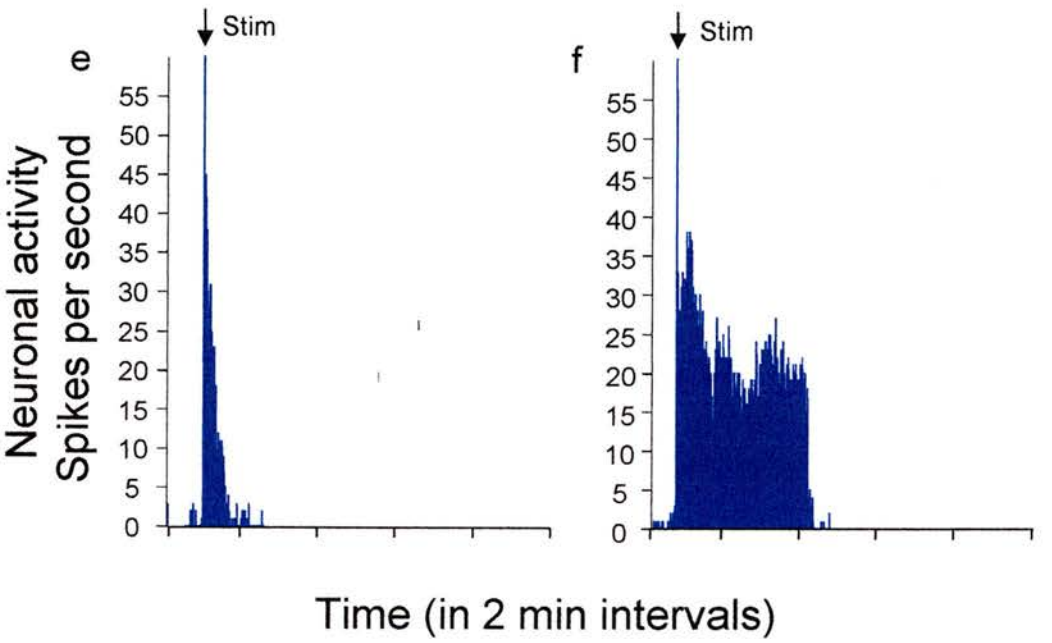

a
b
c
d
Neuronal activity
Spikes per second
Neuronal activity
Spikes per second
0
5
10
15
20
25
30
35
40
45
50
55
0.4g
0.6g
1g
1.5g
2g
4g
6g
9g
12g
15g
Time (in 2 min intervals)

Figure 5.4 (contd)

Typical examples of PSDR activity from spinal dorsal horn and trigeminal neuropathic neurons.

Figure shows initial responses and PSDR activity outlasting the stimulus period for spinal and trigeminal neuropathic neurons. e) spinal dorsal horn neuron exhibiting a short, rapidly degrading activity pattern to a stimulus of 6 grams. f) trigeminal neuron showing prolonged and sustained PSDR pattern also to a stimulus of 6 grams. These results were typical for the neurones that showed afterdischarge (PSDR) in both spinal cord and trigeminal nucleus through a range of stimulus intensities. Although some trigeminal neurones showed briefer afterdischarges, prolonged afterdischarges were never observed in spinal neurons. g) (Inset) Example of an action potential recorded from a single trigeminal neuron. This oscilloscope record shows a typical example of a clearly discriminated action potential. Similar recordings were observed for spinal neurons.


e
f
Stim
Stim
Neuronal activity
Spikes per second
0
5
10
15
20
25
30
35
40
45
50
55
Time (in 2 min intervals)

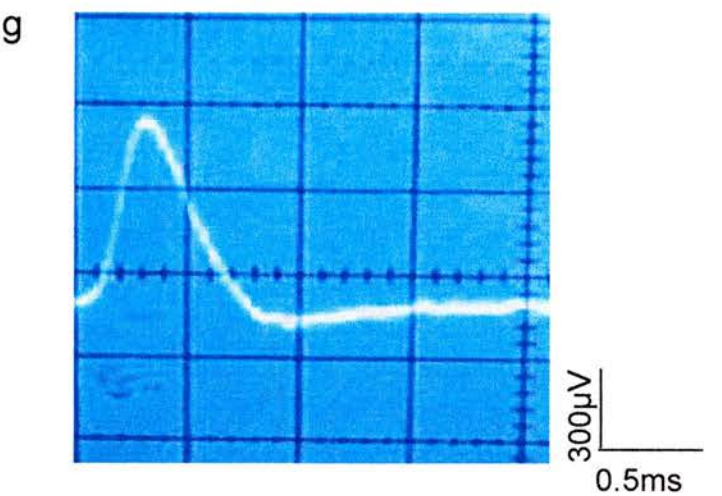
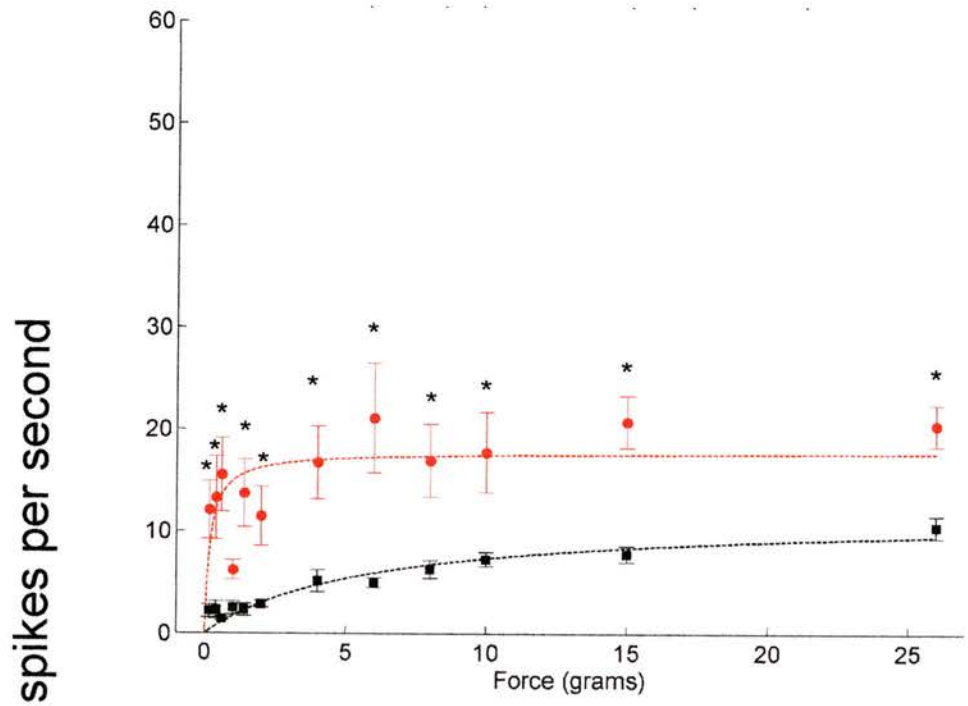

g
300µV
0.5ms

Figure 5.5

Typical examples of stimulus-response characteristics for single spinal dorsal horn and trigeminal caudalis neurons from neuropathic animals.

Figure shows stimulus response curve (derived from data for initial responses only, not including PSDR activity) for normal neurons (black) and neuropathic neurons (red). a) spinal neuropathic neurons have a much increased responsiveness than normal over same force range. b) trigeminal neuropathic neurons show greater responsiveness than normal neurons but to a lesser extent than that seen in spinal neurons. (*) indicates Students'paired t-test. P values, $p<0.05$. Curves were derived by fitting a nonlinear regression using a rectangular hyperbola were (firing rate = (alpha*force)/(force+beta)) where alpha and beta are optimised using a sum-of-least squares metric. This model was used as firing rate will reach maximum level and plateau.

a)


Neuronal activity spikes per second
60
50
40
30
20
10
0
0
5
10
15
20
25
Force (grams)

b)

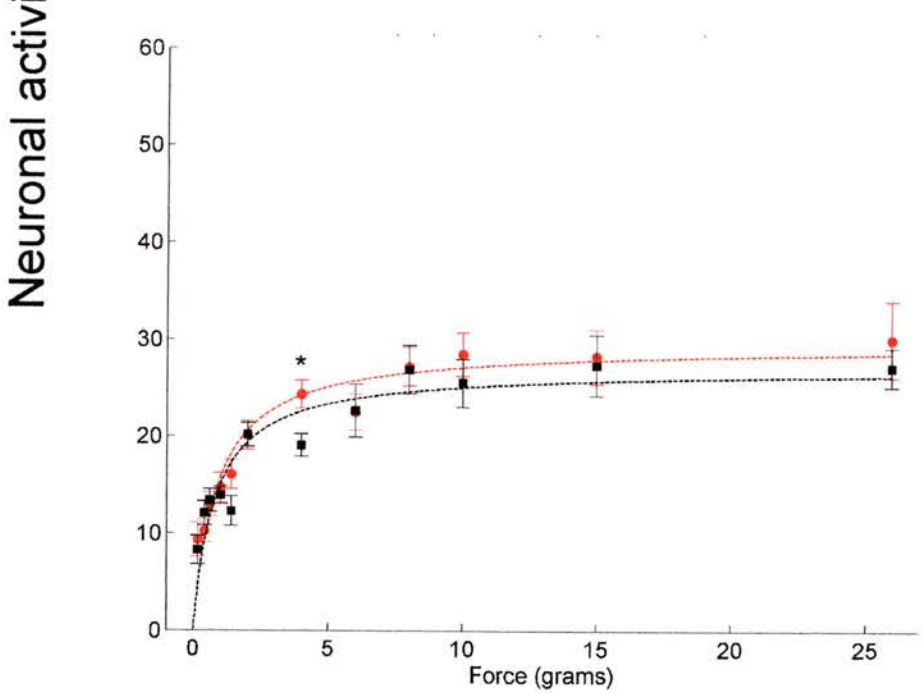

60
50
40
30
20
10
0
0
5
10
15
20
25
Force (grams)

Figure 5.6

Mean total responses of spinal and trigeminal neurons from neuropathic animals.

Shows the total response duration of neurons displaying PSDR at selected von Frey filament forces. Mechanical stimuli were applied at a force equal to half the force needed to elicit a PSDR response from that neuron, the threshold force needed to elicit a PSDR response, and twice the threshold force needed to elicit a PSDR response. Asterisk denotes a statistically significant difference between the mean total response of spinal or trigeminal neurons showing PSDR at twice threshold and the duration of their responses at threshold. (Mann-Whitney U-test, $p<0.05$).

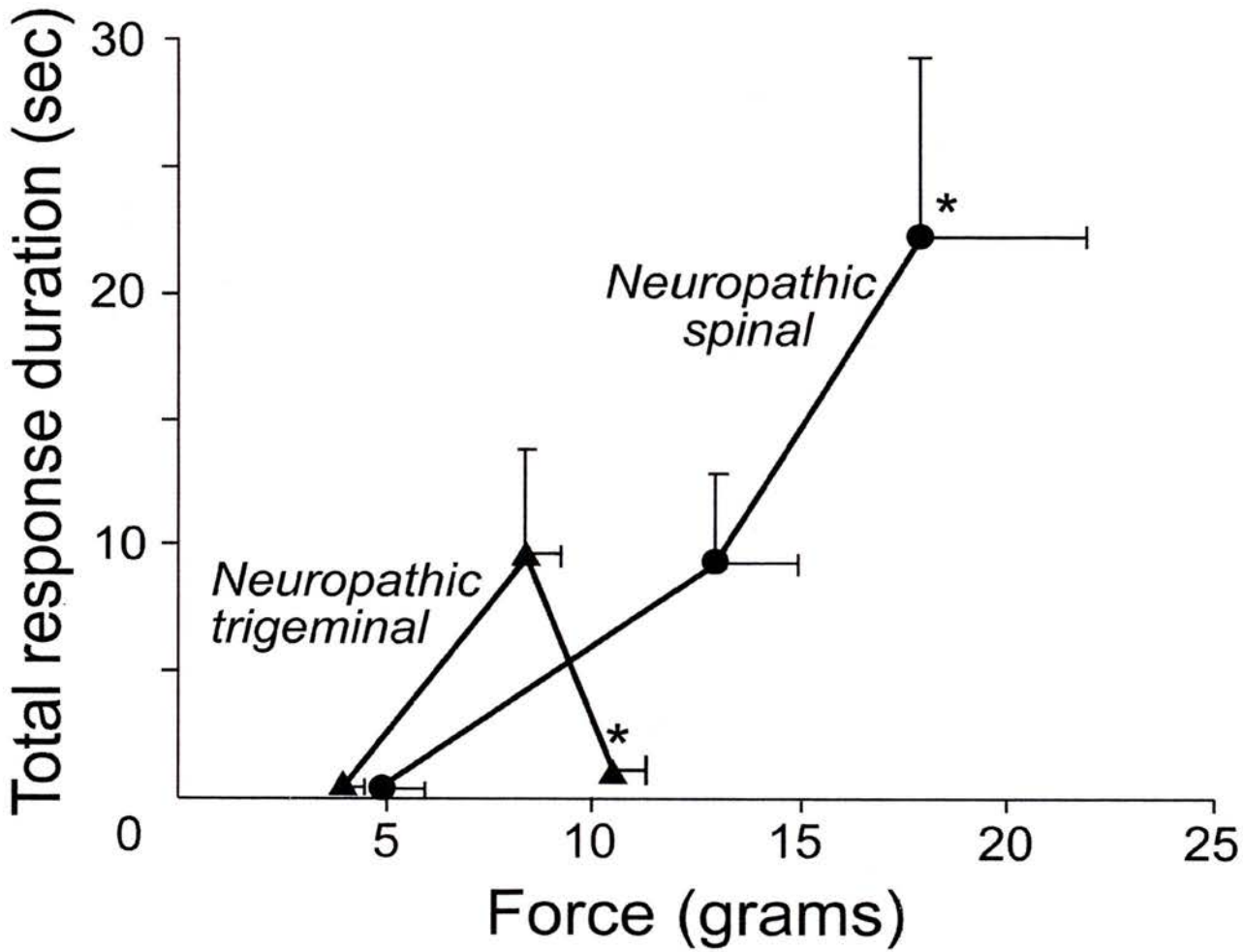

Total response duration (sec)
30
20
10
0
5
10
15
20
25
Force (grams)
Neuropathic
spinal
Neuropathic
trigeminal
*
*

Figure 5.7

Examples of sub division of stimulus-response characteristics for single spinal dorsal horn and trigeminal caudalis neurons from neuropathic animals.

Graphs of neuronal activity (initial responses only) recorded from sites in (a) the spinal cord and (b) trigeminal caudalis. Neurons recorded in naïve animals shown in black, neurons recorded in neuropathic animals shown in blue for after-discharging neurons and red for non after-discharging neurons. (†) and (*) indicates statistically significant differences between responses of neuropathic and naive animals Student's t-test. P values, $p<0.05$. Curves were derived by fitting a nonlinear regression using a rectangular hyperbola were (firing rate = (alpha*force)/(force+beta)) where alpha and beta are optimised using a sum-of-least squares metric. This model was used as firing rate will reach maximum level and plateau.

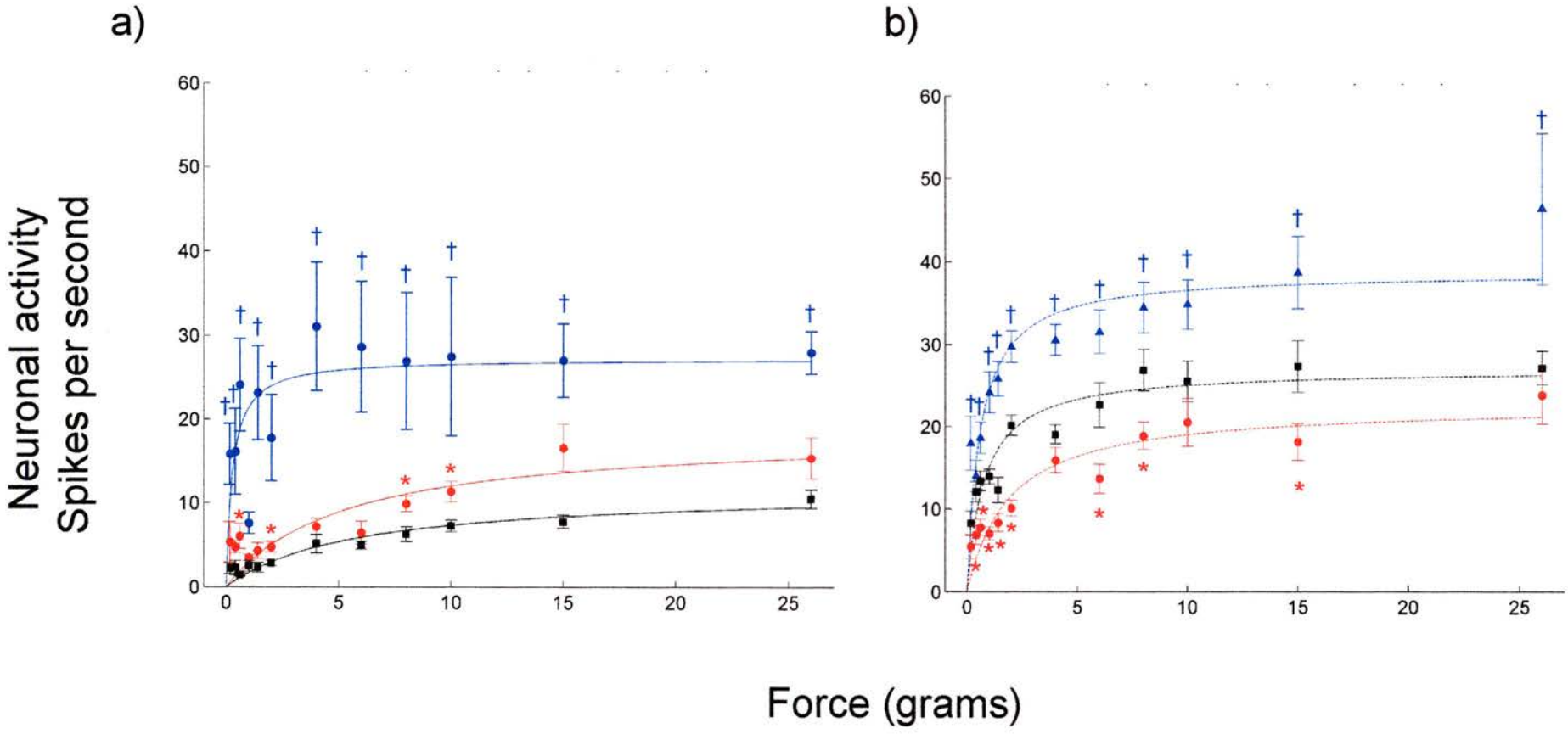
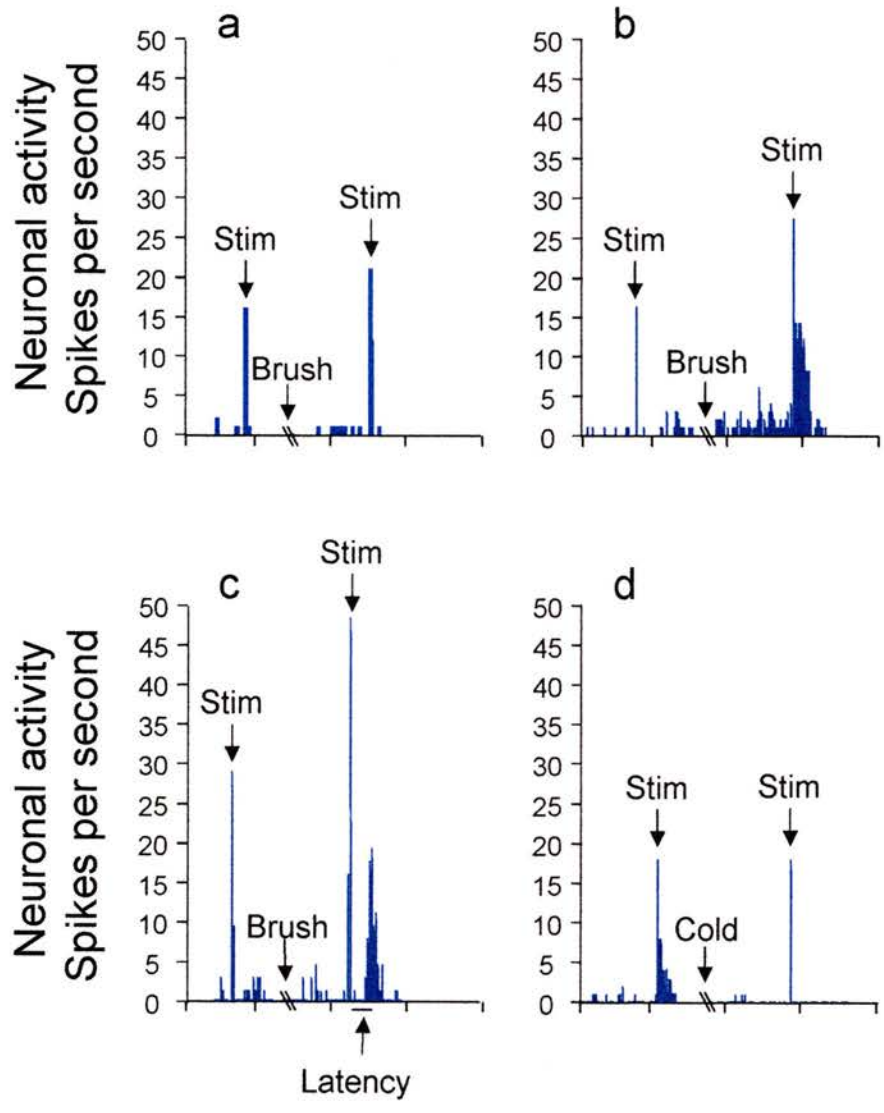

a)
b)
Neuronal activity
Spikes per second
Force (grams)
0
10
20
30
40
50
60
0
5
10
15
20
25

Fig 5.8

Typical examples of electrophysiological recordings of single trigeminal caudalis neurons to show acute induction of sensitisation in their stimulus-evoked responses in neuropathic, but not naïve animals following brush or cold (4°C) stimulation to the face.

(a) shows that in control conditions, in naïve rats, there is no increase in the initial responsiveness of neurons following brush stimulation to the face. However in (b), following development of peak neuropathic behavioural changes after CCI surgery, there is induction of further sensitisation of acute responses in the mechanical stimulus-induced stimulation using von Frey filaments following brush stimulation of the face. Greater responsiveness following brushing of the face was also observed in neurones where the response outlasted the stimulus duration. (c) Furthermore, the development of a latency period (20 sec here) between the initial and PSDR part of the response was only observed following acute brush stimulation of the face. (d) Acute sensitisation was not evoked by all types of sensory stimulation of the face in neuropathic animals, as no statistically significant increases in responses were observed following cold stimulation of the face.

Von Frey forces (1 sec duration) were identical in pre- or post-face stimulus tests. The duration of the brush or cold face stimulus was 5 sec and post-stimulus tests were conducted 2 min later.


a
b
c
d
Neuronal activity
Spikes per second
Stim
Brush
Cold
Latency
Time (in 2 min intervals)

5.3.9 Discussion

From the outset of this discussion it must be reiterated, that although the models are similar in basic principle, the proportion of relevant afferents affected and the functional consequences of the lesion may well differ. The magnitude and composition of the reflex responses are also clearly different between the spinal and trigeminal models. As such, results from the two models are not directly comparable, at least in absolute, quantitative terms. However, the trends between these two models and the trends between naïve and neuropathic animals within each group can be compared.

Taking this in to consideration we have demonstrated that in neuropathic animals there is a shift in the EC50 value of the initial responsiveness of neurons from neuropathic animals to mechanical stimuli from that in the naive state. Furthermore, this shift in EC50 value, differs in magnitude between trigeminal and spinal neuropathic recordings.

We have also demonstrated that some neuropathic recordings can display PSDR activity to forces above a certain threshold level in some applications of von Frey filaments and that this outlasts the inducing stimulus and decays on its own without further interfering stimuli. Moreover, this PSDR activity also differs in magnitude and duration between trigeminal and spinal neurons but does not differ in rate of activity between spinal and trigeminal neuropathic recordings.

It can be seen that trigeminal neuropathic recordings, that already show a facilitated response to mechanical stimuli in their sensitised state, can exhibit a further enhancement of responses after a preceding conditioning mechanical brushing stimulus. This further enhancement does not occur after a preceding conditioning cold stimulus, and in fact, with regard to PSDR activity this cold stimulus appears inhibitory. Some neuropathic trigeminal neurons also exhibited PSDR activity following a mechanical stimulus sometime after the initial response had returned to baseline levels. This latent PSDR activity was seen in the absence of any other

confounding stimulus and was of a considerably larger magnitude and rate than any spontaneous (background) activity present. Interestingly, this latent PSDR response was only ever observed following a preceding conditioning brush stimulus.

Some authors have previously described changes in trigeminal caudalis neuron activity in response to conditioning stimuli. In a model of headache pain, (Burstein et al, 1998), where caudalis neurons that share primary afferent innervation from both the dura of the cranial meninges and a receptive field on the face, application of chemical inflammatory irritants to the dura enhanced activity of caudalis neurons to mechanical stimuli of the face. These neurons also exhibited a long period of discharging activity, which outlasted the stimulus application of chemical irritants. This was very similar in nature to that observed in some neurons in the present experiments and in some cases lasted several minutes, however no period of latency between the stimulus end point and post stimulus discharging activity was reported by the authors in this experiment, in contrast to the distinct and profound latency periods observed in our present data. The experimental field of headache and migraine research however is very large and out-with the scope of this thesis, and direct comparisons cannot be made with the models used in the present experiments but it can verify that trigeminal caudalis neurons are indeed capable of the sort of prolonged activity seen in some of our present experiments.

However, both the location and mechanism of this phenomenon probably differ from that underlying sensitisation in the present study. For example, unlike central sensitisation, the dorsal root reflex has been shown to be NMDA receptor independent (Cervero and Laird, 1996), so these early findings do not relate directly to the results of the current study.

Our results suggest that trigeminal neurons exhibit an ability to acutely sensitise to mechanical stimuli even in an already chronically sensitised state brought about by peripheral nerve damage.

Furthermore, the observation that PSDR activity can follow a period of latency has an important clinical correlate in the condition of trigeminal neuralgia, where just such a period of latency of between 2-60 s following a trigger stimulus is seen before the onset of a painful attack and is characteristic and indeed necessary for a diagnosis

of trigeminal neuralgia (Dubner, 1987). The further observation that trigeminal neurons showing PSDRs exhibit a decrease in their total response to mechanical forces of twice the threshold force needed to elicit a PSDR, is also in agreement with clinical findings which show paroxysmal neuralgic attacks occurring after light touch or pressure to a trigger area but not after noxious pinching or heavy pressure (Sharav, 2002). Taken together, these observations suggest that the infraorbital nerve chronic constriction injury model of trigeminal neuropathic pain may more closely resemble that of trigeminal neuralgia than previously thought, (Rappaport and Devor, 1994).

In both the spinal dorsal horn and trigeminal caudalis, circuitry that underlies the transmission of somatosensory information from the receptive fields of the hind paw or the side of the face is changed in some way to create a facilitated transmission state following nerve damage. Through whatever mechanism this facilitation occurs, be it changes in synaptic strength, new synapse formation, summation of information from nociceptors and non-nociceptors, recruitment of more cells into a local network, altered descending influences, or combinations of all of the above, in the trigeminal system at least, the potential is still available for further increases in responsiveness. The increase in both initial and PSDR responses evoked by mechanical stimuli following a conditioning brush stimulus or the apparent reduction of mechanically evoked PSDR activity following a conditioning acute cold stimulus, suggest that this sensitised state has not yet reached saturation and is still capable of up or down regulation.

Indeed this modulation that the circuitry is still subject to may be dynamic and complex. The enhanced facilitation or acute sensitisation seen in addition to the chronically sensitised state is only seen in these experiments after conditioning brush stimuli. However, in some cases this enhancement is only evident after a period of latency. This latency could well be caused by a temporary period of inhibition following the mechanical excitatory stimulus. Whether this is the case or not can not be gleaned from these results but the possibility exists that acute sensitisation may be affecting both excitatory and inhibitory mechanisms at the same time.

Wind-up, the frequency-dependant steady increase in C-fibre activity seen in response to application of repetitive (usually electrical) stimuli of constant intensity,

is another form of facilitation shown in dorsal horn and trigeminal complex neurons (Mendell and Wall, 1965; Mendell, 1996; Herrero et al, 2000; Dallel et al, 1999; Parada et al, 1997) and is known to be NMDA receptor dependant (Sullivan and Dickenson, 1987; Davis and Lodge, 1987; Luccarini et al, 2001; Woda et al, 2001). However, recently Woda et al, (2004) have shown in the trigeminal system (utilising the unique indirect C-fibre input to deep WDR neurons in the subnucleus oralis from the superficial laminae in the caudalis and recording from both these sites), that the application of the NMDA antagonist AP-5 in the SpO acts to reduce the wind-up that can be induced, but that the same antagonist in the SpC actually increases wind-up observed in the WDR neurons of the SpO. These results suggest that NMDA receptors have differing actions in discrete areas of the trigeminal system and, it is argued by the authors, in discrete laminae in the dorsal horn, with NMDA receptors in the superficial caudalis acting to actually restrain wind-up. This new assertion that NMDA receptors may be involved in opposing fashions depending on location may well necessitate further investigation into NMDA receptor subtype activities.

Wind-up and central sensitisation are not the same (Woolf, 1996) as wind-up will amplify afferent input for only a few minutes while central sensitisation is a long lasting phenomenon. The NMDA-dependant inhibition of wind-up however may prevent further activation of intracellular signalling cascades involved in the development of central sensitisation under sustained nociceptive C-fibre barrage (Woolf and Salter, 2000). Woda et al, (2004) speculate that under pathological conditions, dysfunction of the NMDA-dependant inhibition of wind-up could contribute to central sensitisation of pain systems.

Given the difference in NMDA receptor subtype expression between these two regions (namely the absence of NR2B subunit in the SpO) then it would seem reasonable to repeat these experiments with agents more selective for NR subunits.

Clinical trials have indicated selective NR2B NMDA receptor antagonists to be effective in spinal neuropathic sensitisation (Boyce et al, 1999a). This is an interesting point as the NR2B subunit is expressed in the trigeminal complex only in the superficial laminae of the SpC, the same place that AP-5 has been shown to increase wind-up. Selective agents may help to unravel the role of these receptors in this region and the connection between wind-up and central sensitisation.

Aβ-fibre input may be important to this sensitisation. In the CCI model the constriction injury causes a partially selective degeneration of large myelinated fibres in the sciatic nerve. This leaves a lot of the large myelinated fibres compromised whilst a good proportion of the small unmyelinated fibres remain intact (Guilbaud et al, 1997). When this occurs, the conduction velocity of these fibres becomes slower, meaning that the normal separation of sensory signals from the fast mechanically sensitive large myelinated fibres to the slower unmyelinated polymodal nociceptors becomes deranged. This results in the sensory information from all these fibres becoming bunched together opening the possibility of summation of this information. But more interestingly these results show that, in some cells, the acute increase in PSDR activity seen following a conditioning brush stimulus actually occurs at forces of <1.2 grams. These forces are outwith the range of forces that evoke a reflex withdrawal in behavioural tests even in neuropathic animals. If this low threshold mechanical stimulus is enough to evoke the PSDR activity at force that may not be noxious, then it is further evidence for a direct contribution of non-nociceptors in this acute sensitisation.

Moreover, the response following a cold conditioning stimulus, which demonstrated an inhibitory effect on PSDR properties of some neurons, will be exerting effects through cold responsive nociceptors. This indicates that the inhibitory influence may be mediated through a different set of peripheral afferent fibres than that of the excitatory response after brush. However the nature of this mechanism is unknown.

Chapter 6

CCI-induced changes in key NMDA receptor and adapter protein expression differ between the spinal cord and trigeminal system

6.1 Aim

The purpose of these experiments was to assess any changes in protein expression in ipsilateral and contralateral sides of the spinal cord and trigeminal complex that might occur after nerve damage to either the sciatic or the trigeminal nerve. This assessment would allow comparison between these regions so that similarities or differences may be analysed. Tissue was taken from the spinal cord and regions in the trigeminal complex in both neuropathic and sham-operated animals. This tissue was examined for changes in protein levels by Western blotting both ipsilateral and contralateral to nerve injury and in control groups.

METHODS

Tissue Preparation for Western Blotting

Animals were anaesthetised with halothane 5% in an oxygen vehicle and appropriate regions of either the spinal cord or brainstem were exposed. In spinal cord preparations, the spinal cord was transacted at the level of the 2^{nd}-6^{th} lumber vertebrae. Then this section of cord was removed and cut in half along its length allowing the separation of ipsilateral and contralateral sides. These were then placed in separate vials for homogenisation. For brainstem preparations, the brainstem from the level of the 1^{st} cervical vertebra to the interaural level was removed and then the oralis, interpolaris and caudalis sections of the brainstem were measured length ways in relation to the point of obex (i.e., oralis= 2.5mm-4mm rostral to obex, interpolaris= 1.0mm-2.5mm rostral to obex and caudalis= obex -2.0mm caudal to obex) and sectioned horizontally. Each of these sections were then measured from

the midline as they lie laterally from this (i.e., oralis=2.5mm-3.5mm lateral to midline and interpolaris and caudalis= 2.0mm-3.0mm lateral to midline) and cut length ways. Finally the ventral 1mm of this tissue was removed to exclude extraneous ventral regions that were not of interest.

Immediately following tissue extraction, samples were individually weighed and transferred to a 1ml homogeniser containing ten volumes of ice cold 2x sample buffer. Once homogenised, samples were heated to 100°C for 5 min. Genomic DNA / RNA was sheared by passing the lysate several times through a narrow gauge hypodermic needle and syringe prior to centrifugation at 13,000 x g for 10 min at room temperature. The supernatant was transferred to a fresh micro-centrifuge tube and either analysed by gel electrophoresis, or stored at -40°C.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was performed using the Tris-glycine discontinuous buffer system described by Laemmli et al, (1970), for protein separation. 12 well 4-12% pre-cast gels were used for the experiments shown. The gel tank was filled with electrophoresis running buffer (1x). Samples and molecular weight marker proteins were loaded into the wells of the stacking gel and electrophoresed at a constant voltage of 200V for approximately 45 minutes until the dye front had run off the end of the resolving gel. Following electrophoresis the protein was transferred to PVDF membrane.

Electrotransfer of Protein from SDS-PAGE Gels to PVDF

PVDF membranes were soaked in methanol for 1 minute. Following SDS-PAGE, the gel, PVDF membrane, pre-cut filter paper, and fibre pads were soaked in SDS buffer for 2 minutes. The Bio-Rad Protein II gel transfer apparatus was then assembled, ensuring no air bubbles were present.

The gel, membrane and filter paper sandwich was then placed in the electrophoresis buffer chamber, along with an ice pack, and filled with transfer buffer. A constant voltage of 30V was applied for 70 min.

Coomassie Staining

Polyacrylamide gel slabs were transferred into an excess volume of Coomassie staining solution and agitated on an orbital mixer for 1 minute at room temperature. The gel was then steeped in a destaining solution until bands became clearly visible. Protein bands could be visualised following several changes of excess Coomassie destaining solution.

Western Blotting

Following electrophoretic transfer, the PVDF membrane was rinsed briefly in PBS tween (PBS-T). Then blocked in 4% Marvel dried skimmed milk in PBS-T for either 60 minutes at room temperature or overnight at 4°C on a rocking platform. Following blocking, the membrane was briefly rinsed with PBS-T and incubated with the primary antibody at the required dilution in PBS-T with 4% Marvel at room temperature for 90 minutes. After six, 5 minute washes in PBS-T at room-temperature, the PVDF was incubated with the Horseradish peroxidase (HRP)-conjugated secondary antibody in PBS-T with 4% Marvel for 50 minutes. Finally the PVDF was washed as above, with the addition of a final rinse in PBS only.

Enhanced Chemiluminescence Detection

To detect bound antibody, membranes were incubated with a minimal volume of enhanced chemiluminescence (ECL) detection reagent for 1 minute at room temperature, as described in the manufacturer's protocol. PVDF was placed between two transparency sheets before exposure to Hyperfilm ECL autoradiography film. Several exposures were carried out to ensure linear exposure for direct comparison between bands.

Analysis of relative grey levels

Exposed films were scanned at 600dpi resolution and densiometric analysis performed via computer with scan analysis software (Scion image GelPro; NIH, USA). All densities were relative to housekeeping protein levels and all samples that

were compared were run on the same gels or, at the very least, run under similar conditions. A minimum number of three samples (one sample from each rat) for each experiment were examined.

6.2 Results

In order to accept changes as a consistent response to nerve injury, it was required that a similar pattern of change compared to controls would be seen in at least 3 separate animals. In animals taken at peak neuropathic change, as shown by behavioural testing, the levels of NMDA receptor subunits and the MAGUK family of adapter proteins were examined by Western immunoblotting. Expression levels were compared on the same blots with those of the ubiquitous housekeeping enzyme, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described in Moss et al 2002, which does not change in expression following a wide range of afferent manipulations. Figures 6.1 and 6.2 show examples of immunoblots from nerve-injured animals compared to sham-operated controls. Following ECL visualisation of immunoreactive proteins, quantitative densitometry of the proteins of interest relative to GAPDH expression was used to quantify the relative extent of any changes seen.

6.2.1 NMDA receptor subunit expression in the spinal cord and trigeminal complex, and its response to nerve injury.

In Figure 6.1, Western blots show that NR1, NR2A and NR2B subunits are all expressed in the spinal cord and in at least some regions of the trigeminal complex. Notably, the NR2B subunit is not expressed in the oralis of the trigeminal complex, although it is expressed in the caudalis and the spinal cord.

There was no change in NR1 subunit expression in either the oralis or caudalis regions of the trigeminal complex following CCI, whilst in spinal cord NR1 subunit expression is seen to decrease ipsilateral to nerve injury as compared to sham-operated controls (Table 6.1). The reduction in NR1 expression in the spinal cord represents a change of -11.1 grey scale units ipsilateral to CCI compared with the contralateral side, which had a grey scale score of 41.8 ± 1.5; mean ± SEM (i.e. a

mean reduction of 27%, $p<0.02$, Paired Student's t-test n=5). No change in NR2A expression was seen in any of the trigeminal regions or the spinal cord, whereas in both regions NR2B expression is seen to increase ipsilateral to CCI. This change in NR2B expression in the trigeminal caudalis is an increase of 18.4 grey scale units with grey scale scores of 61.5 ± 7.4 ipsilateral and 43.1 ± 7.4 contralateral to CCI. In the spinal cord the increase represents a rise of 12.1 grey scale units (with grey scale scores of 52.3 ± 2.9 ipsilaterally and 40.4 ± 3.0 contralateral to injury). These changes represent ipsilateral increases of 41% in trigeminal caudalis ($p<0.02$, Paired Student's t-test n=4) and 30% in spinal cord ($p<0.03$, Paired Student's t-test n=5). The NR2C subunit is very diffusely expressed throughout the entire trigeminal complex (Watanabe et al 1994), but in the present experiments was not investigated as there is no evidence to date to implicate it in neuropathic pain states. NR2D expression, however is localised to a discrete area of the caudalis region adjacent to, and slightly overlapping, the area of NR2B subunit expression (Watanabe et al, 1994). This subunit was also investigated, but due to the lower affinity and specificity of the antibody available for study, results were inconclusive.

6.2.2 MAGUK expression in the spinal cord and trigeminal complex and its response to nerve injury.

The MAGUK proteins (PSD-95, SAP-97, SAP-102, and Chapsyn-110), known to be associated with the NMDA receptor, were shown by Western blotting to be expressed in spinal cord and in both the oralis and caudalis regions of the trigeminal complex (Figure 6.2). Following CCI, there was no change in SAP-97 or SAP-102 expression in either the spinal cord or trigeminal system (Table 6.2 and example blots shown in Figure 6.2). PSD-95 however, which showed no change in expression in the oralis or caudalis regions of the trigeminal complex following CCI, does increase in expression ipsilateral to nerve injury in the spinal cord (Table 6.2). The increase in PSD-95 expression in the spinal cord represents a change of 31 grey scale units ipsilateral to CCI compared with the contralateral side, (grey scale scores were 53 ± 9.3 and 22 ± 4.4; mean ± SEM for ipsilateral and contralateral respectively), an increase of 140% ($p<0.02$, Paired Student's t-test, n=5). Furthermore, the ratios of

PSD-95 expression relative to GAPDH in the spinal cord under equivalent conditions are around five times less than those seen in the trigeminal caudalis and about half that seen in the oralis. Assuming that levels of GAPDH are reasonably consistent through these two sensory regions, this may indicate that PSD-95 expression is constitutively higher in the trigeminal complex than the spinal cord. Chapsyn-110, which showed no demonstrable change in expression in the spinal cord does exhibit a marked 40% decrease in ipsilateral expression in the trigeminal caudalis following CCI (with relative grey scale scores of 18 ± 3.5 and 30 ± 5.7 mean ± SEM for ipsilateral and contralateral respectively ($p<0.02$, Paired Student's t-test, $n=3$). The distribution of MAGUK proteins in the trigeminal complex has, as yet, not been investigated by Immunohistochemistry.

Figure 6.1

Immunoblots of NMDA receptor subunit expression in trigeminal oralis and caudalis, and in spinal cord in CCI treated animals and sham-operated controls.

Western immunoblots of NR1, NR2A and NR2B subunit expression in spinal cord and trigeminal nuclei. Results are typical of n=3 minimum in each case. Changes in protein expression between ipsilateral and contralateral sides in CCI animals can be seen as differences in blot density. NR1 subunit expression is seen to decrease ipsilateral to injury in the spinal cord but not in the trigeminal complex. NR2A expression remains unchanged following injury throughout. NR2B expression is increased ipsilaterally in both the trigeminal caudalis and the spinal cord, and this subunit is not expressed at detectable levels in the trigeminal oralis.

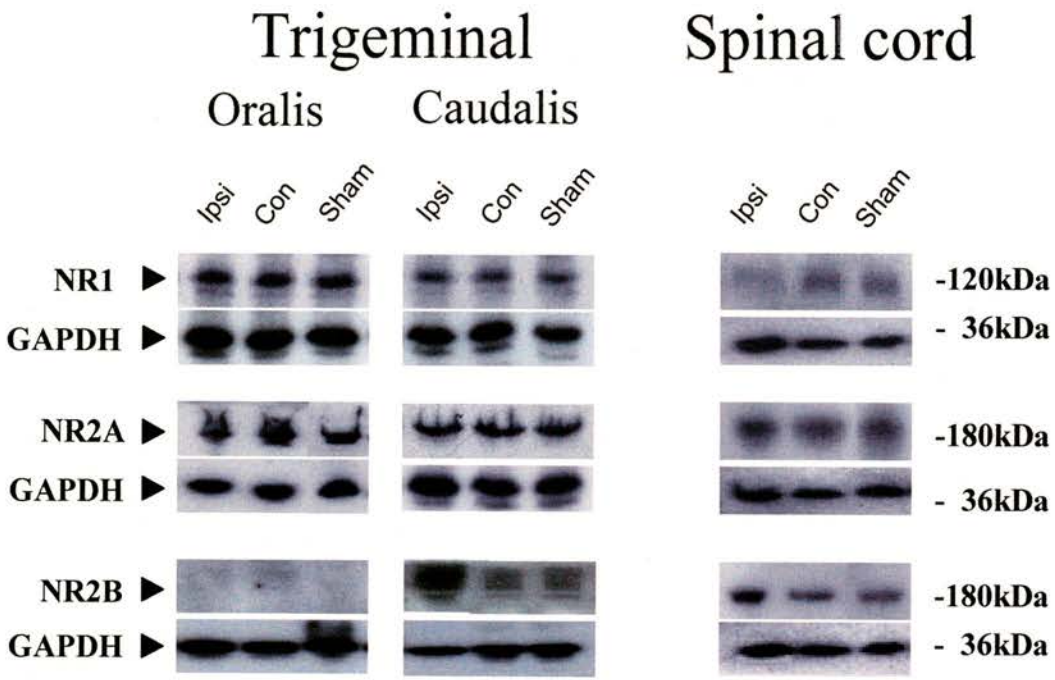

Trigeminal
Spinal cord
Oralis
Caudalis
Ipsi
Con
Sham
Ipsi
Con
Sham
Ipsi
Con
Sham
NR1
GAPDH
NR2A
GAPDH
NR2B
GAPDH
-120kDa
- 36kDa
-180kDa
- 36kDa
-180kDa
- 36kDa

Figure 6.2

Immunoblots of MAGUK protein expression in trigeminal oralis and caudalis, and in spinal cord in CCI treated animals and sham-operated controls.

Immunoblots of MAGUK proteins ipsilateral and contralateral to CCI at peak neuropathic behavioural change and for sham operated animals. Results are typical of n=3 minimum in each case. Changes in protein expression between sides in CCI animals can be seen as differences in blot density. PSD 95 is shown to increase ipsilateral to injury in the spinal cord, but no change is seen in the trigeminal complex. Chapsyn 110 shows a relative decrease of expression ipsilateral to injury in the trigeminal caudalis but not in oralis or the spinal cord. SAP 97 and SAP 102 remain unchanged in regions investigated.

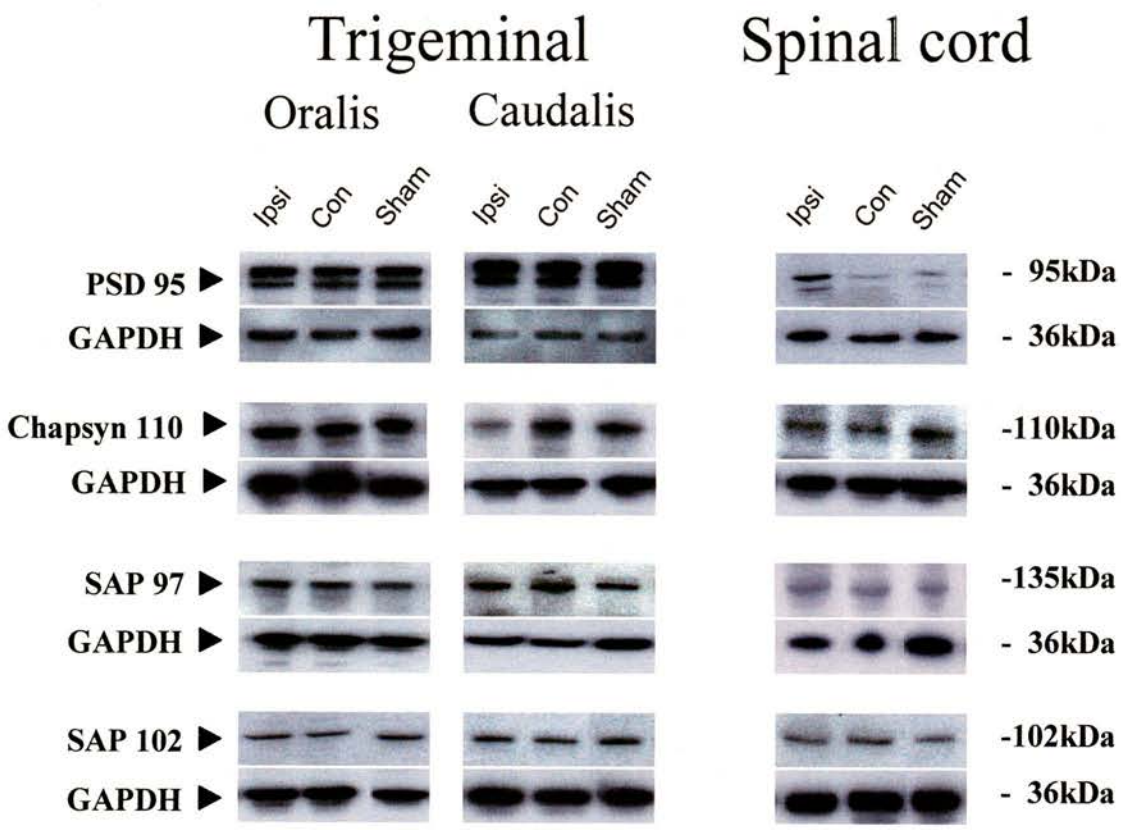

Trigeminal
Spinal cord
Oralis
Caudalis
Ipsi
Con
Sham
Ipsi
Con
Sham
Ipsi
Con
Sham
PSD 95 ▶
GAPDH ▶
- 95kDa
- 36kDa
Chapsyn 110 ▶
GAPDH ▶
-110kDa
- 36kDa
SAP 97 ▶
GAPDH ▶
-135kDa
- 36kDa
SAP 102 ▶
GAPDH ▶
-102kDa
- 36kDa

Table 6.1

NMDA receptor subunit expression in trigeminal nucleus oralis and nucleus caudalis, and in spinal cord in CCI treated animals and sham-operated controls.

Table shows GAPDH normalised relative grey scale scores ± the SEM for protein analysis of NMDA receptor subunits ipsilateral and contralateral to CCI at peak neuropathy and for sham operated animals (n=3 minimum in each case). Statistically significant changes in protein expression ipsilateral to CCI are indicated as * $p<0.05$ compared to contralateral CCI (Paired Student's t test) and † $p<0.05$ compared to sham controls (Unpaired Student's t test). NR1 subunit expression decreases ipsilateral to injury in the spinal cord but not the trigeminal complex. NR2A expression remains unchanged throughout. NR2B expression is increased ipsilateral to injury in both the trigeminal caudalis and the spinal cord, and is not expressed in the trigeminal oralis.

Expression of protein as mean ± SEM of % GAPDH expression									
	Trigeminal complex						Spinal cord		
	Oralis			Caudalis					
Protein	Ipsi CCI	Cont CCI	Sham	Ipsi CCI	Cont CCI	sham	Ipsi CCI	Cont CCI	sham
NR1	18.5 (± 10.3)	17.3 (± 12.1)	20.0 (± 5.4)	21.0 (± 9.0)	26.0 (± 2.0)	24.0 (± 6.8)	30.7 * (± 1.9)	41.8 (± 1.5)	44.3 † (± 2.0)
NR2A	73.1 (± 16.9)	84.7 (± 24.6)	78.3 (± 18.6)	75.7 (± 10.9)	71.5 (± 17.7)	69.9 (± 15.4)	48.6 (± 2.7)	51.3 (± 3.1)	47.2 (± 1.6)
NR2B	-	-	-	61.5 * (± 7.4)	43.1 (± 7.4)	41.2 † (± 5.0)	52.3 * (± 2.9)	40.4 (± 3.0)	39.8 † (± 2.1)

Table 6.2

MAGUK protein expression in trigeminal nucleus oralis nucleus and caudalis, and in spinal cord in CCI treated animals and sham-operated controls.

Table shows GAPDH normalised relative grey scale scores ± the SEM for protein analysis of MAGUK proteins ipsilateral and contralateral to CCI at peak neuropathy and for sham operated animals animals (n=3 minimum in each case). Statistically significant changes in protein expression ipsilateral to CCI are indicated as * $p<0.05$ compared to contralateral CCI (Paired Student's t test) and † $p<0.05$ compared to sham controls (Unpaired Student's t test). PSD 95 is shown to increase ipsilateral to injury in the spinal cord but no change is seen in the
trigeminal complex. Chapsyn 110 shows a decrease of expression ipsilateral to injury in the trigeminal caudalis but not in oralis or the spinal cord. SAP 97 and SAP 102 both remain unchanged in the regions investigated.

Expression of protein as mean ± SEM of % GAPDH expression									
	Trigeminal complex						Spinal cord		
	Oralis			Caudalis					
Protein	Ipsi CCI	Cont CCI	sham	Ipsi CCI	Cont CCI	Sham	Ipsi CCI	Cont CCI	sham
PSD 95	65.8 (± 10.2)	71.1 (± 13.4)	51.3 (± 8.1)	127 (±12.9)	121 (± 1.9)	146 (± 19.5)	53.3 * (± 9.3)	22.5 (± 4.4)	18.1 † (± 4.0)
Chapsyn 110	35.5 (± 5.1)	32.6 (± 10.5)	35.2 (± 6.0)	18.8 * (± 3.5)	30.9 (± 5.7)	33.2 † (± 9.3)	56.6 (± 1.9)	52.3 (± 2.2)	50.4 (± 4.8)
SAP 97	66.4 (± 4.0)	65.5 (± 10.2)	48.4 (± 3.4)	70.9 (± 8.2)	68.3 (± 15.7)	61.8 (± 6.3)	61.3 (± 3.4)	59.7 (± 4.0)	62.6 (± 3.8)
SAP 102	24.2 (± 9.7)	24.5 (± 8.2)	23.7 (± 7.1)	46.1 (± 13.4)	40.7 (± 15.1)	38.4 (± 11.7)	30.0 (±2.1)	29.7 (± 5.2)	32.6 (± 1.6)

6.3 Discussion

As mentioned previously (in section 1.10.3) the NMDA receptor (NMDAR) was demonstrated to be central to both the phenomenon of central sensitisation in the spinal cord and has been shown to be crucial to the development of pain behaviours in both the spinal cord and the trigeminal caudalis and oralis, despite having no role in acute nociception (Woolf, 1983; Mayer et al, 1999; Woda et al 2000; Park et al, 2001).

In the introduction to this thesis it was mentioned that the combination of NR1 subunit is obligatory in NMDA complexes and combines with one of the four types of NR2 subunits. The different types of NR2 subunit confer different qualities on the pharmacodynamic and biophysical properties of the channel (Monyer et al., 1992; Flint et al., 1997; Vicini et al., 1998; Quinlan et al., 1999). For example, NR2A-containing NMDARs mediate shorter excitatory postsynaptic currents than those containing NR2B, and NR1/NR2A assemblies are less sensitive to glutamate than other heteromeric channels. As can be seen, in light of these differences it may be considered that any change in the balance of NR2 subunit ratio may affect the overall functioning of the synapse. Indeed in memory studies in the piriform cortex, rule learning regulates the composition of synaptic NMDARs, resulting in receptors with a higher complement of the NR2A subunit protein relative to NR2B. Rule learning also reduces long-term potentiation (LTP) induced by high-frequency stimulation of the intracortical axons in piriform cortex slices (Quinlan et al 2004).

In our experiments we have found that using Western blotting following CCI, a significant rise in ipsilateral NR2B subunit protein expression was detected ipsilateral to injury in both spinal cord and trigeminal regions, whereas no change in NR2A was detected. Also evident in our experiments was a decrease in NR1 protein expression ipsilateral to nerve injury in the spinal cord but interestingly a static expression in the trigeminal system.

Previous researchers using another peripheral nerve damage model, have shown with single-cell PCR, that fewer rat dorsal horn neurons expressed NR2A mRNA compared with controls 1–2 weeks after L5 spinal nerve transection (Karlsson et al, 2002). As NR2B was the subunit most commonly expressed in rat dorsal horn, the reduction of NR2A expression after a peripheral nerve lesion could make the contribution of the former subunit proportionally larger. Indeed, the observed changes in the dose-response curve of NMDAR currents were consistent with a relative increase in NR2B expression.

These results reflect the known differential involvement of the NMDA receptor complex in inflammatory and neuropathic pain (Garry et al, 2003) and furthermore indicate possible differing contributions of NMDAR subtypes in distinct models of neuropathic pain itself. However, the common factor between these two experiments based on neuropathic pain models is that they both show a relative increase in NR2B subunit ratio compared to NR2A.

Some authors have suggested the importance of the NR2B but not the NR2A subunit in neuropathic pain conditions. Also, the relative increase in NR2B, NR2C, and NR2D populations could well be part of an adaptive or "fail safe" response that would serve to enhance NMDAR-mediated currents, thereby ensuring the adequacy of neurotransmission compromised by nerve damage (Karlsson et al, 2002; McCormack, 1999). The particular importance of NR2B subunits in mediating pain is further substantiated by the fact that changes in pain-related behaviours in NR2A subunit knockout mice (compared with wild type controls) have not been demonstrated in several acute and chronic pain models (Petrenko et al, 2003b).

As the NR1 subunit is obligatory for functional NMDARs, an increase in NR2B would increase the likelihood of remaining NR1 subunits forming receptors with NR2B. Furthermore, a decrease in NR1 subunit expression combined with an increase in the NR2B expression and no change in NR2A expression would increase the likelihood of remaining NR1 subunits forming receptors with NR2B and could in effect reduce the amount of NR2A containing NMDARs at the synapse allowing NR2B containing NMDAR synaptic insertion.

NR2B containing NMDARs have been shown to be primarily situated in the extrasynaptic regions of substantia gelatinosa neurons of the dorsal horn in the normal state (Momiyama, 2000). However, following nerve injury with associated upregulation of NR2B subunit expression, this presentation might well change recruiting more NR2B-containing NMDARs into the synapse. This change to a functionally greater role of NR2B seems likely because no difference in pain behaviours has been detected between normal mice and NR2A-deficient mice (Petrenko et al, 2003a).

An increase in NR2B subunit expression alone has been shown not to be sufficient for the increased insertion of NR2B containing NMDARs into the synapse (Barria and Malinow, 2002), whereas an increase in NR2A subunit is sufficient to increase NR2A-containing NMDARs in the synapse. A decrease in the proportion of NR2A NMDARs may facilitate the synaptic presentation of NR2B-containing NMDAR, but so may other factors such as EphB receptor activation (Dalva et al, 2000), which is known to associate with the NMDAR and PSD-95 (Bruckner and Klein 1998; Garner et al. 2000) and has recently been implicated in chronic pain states (Battaglia et al, 2003)

However, in the trigeminal complex NR2B-containing NMDARs may well be crucial to plasticity seen in the caudalis region but can not be involved in plasticity seen in the oralis. Therefore, other mechanisms must be fulfilling this role in any plasticity that may occur in this region. These might involve NMDA receptors containing NR2 subunits other than NR2B or alternative mechanisms that rely on receptors other than the NMDA receptor, potentially including other types of excitatory amino acid receptor such as AMPA/kainite type glutamate receptors.

Many small-diameter primary afferent fibres terminating in the dorsal horn express NMDARs, and activation of presynaptic NMDARs causes the release of substance P from primary afferents (Liu et al, 1997). NR2B is predominantly expressed on small-diameter primary afferents (Ma and Hargreaves, 2000). Although the direct presence of presynaptic NMDA autoreceptors in the trigeminal nuclei has not been demonstrated, the action of dynorphin A on substance P (SP) release from trigeminal caudalis primary afferents has been shown to be affected by NMDA antagonists,

indirectly indicating their presence (Arcaya et al, 1999). In addition, glutamate released from the presynaptic terminal can potentially enhance its own release in a feed-forward manner in response to subsequent stimuli. Rat DRG neurons contain NR1 and NR2B but not NR2A (Marvizon et al, 2002). Therefore, because SP, calcitonin gene-related peptide and glutamate co-occur in small diameter primary afferent terminals, presynaptic NR2B-containing NMDARs may facilitate and prolong the transmission of nociceptive messages through the release of these neurotransmitters. Therefore NR subunit changes may not necessarily involve complexes that are directly involved in postsynaptic sensitisation; some changes may reflect a dynamic regulation at presynaptic terminals as well.
Due to the lack of NR2B expression in the oralis region this would indicate a possible difference in function of the NMDA at the presynaptic terminal as well.

Also, in the spinal cord, the effect of nerve damage may greatly influence the distribution of fibres throughout the segments associated with that nerve. In the trigeminal complex however, although the infra-orbital nerve may be affected, the complex still receives afferent innervation from all the remaining branches of the trigeminal nerve (see Sessle, 2000). This may mean that the levels of NR1 in the complex may be less obviously affected by the injury because changes may be more diffusely spread out through the complex and therefore less detectable.

In recent years the NMDAR has been shown to be part of a larger complex that has been postulated to include over 70 proteins (Husi et al, 2000), showing the actual coupling of the receptor with (amongst others) metabotropic glutamate receptors (mGluR) receptors (Tu et al, 1999) which may regulate other proteins by the phosphorylation they induce. Also in this protein complex there are several signalling molecules which are thought to form links to or direct components of signalling pathways such as the Ras-MAPK pathway (Grant and O'Dell, 2001). PSD-95 is important because it links several of these pathways to the NMDAR (Kim et al, 1998; Chen et al 1998; Westphal et al, 1999; Colledge et al, 2000), through appropriate PDZ target motifs (Christopherson et al 1999, Kornau et al 1995). PSD-95 has been shown to be necessary for NMDA mediated thermal hyperalgesia (Tao

et al 2000) and also for the induction of pain behaviours following neuropathic injury, but not inflammatory insult, to the sciatic nerve (Garry et al 2003).

The experiments presented here have demonstrated an increase in PSD-95 expression in the spinal cord on the ipsilateral side following CCI nerve injury to the sciatic nerve and show that this increase is concomitant with an increase in the NR2B NMDAR subunit (a subunit which binds PSD-95) in the same region.

The rise in NR2B subunit and PSD-95 protein expression at a time concomitant with the development of neuropathic pain behaviours is consistent with previous reports showing the necessity for an intact NR2B-PSD-95 complex for the development of neuropathic behaviours in PSD-95 mutant mice (Garry et al, 2003). Also shown, was the significantly greater activation of CaM kinase II ipsilaterally in wild-type animals following CCI but not in mutant animals. Cam kinase II inhibitors clearly reversed neuropathic thermal hyperalgesia and mechanical allodynia seen in wild-type mice following CCI whereas a control or vehicle did not. Recent studies indicate that calcium/calmodulin stimulation induces auto-phosphorylation of CaM kinase II and docking of the active kinase to the C-terminal domain of NR2B (Bayer et al, 2001). Although the spinal complement of CaM kinase II was shown to be able to respond to calcium elevating stimuli, the NMDA receptor-mediated calcium entry that occurs physiologically during CCI appears to activate CaM kinase II more effectively in wild-type than in PSD-95 mutant mice. Therefore PSD-95 seems to play an important role in facilitating the functional coupling between the NMDA receptor and CaM kinase II in CCI.

In the spinal cord this increase in PSD-95 may also indicate a specific induction of protein expression which may act to reduce the amount of NR2B-mediated NMDAR internalisation (Levazzarri et al, 2003) or interaction with EphB receptors (Battaglia et al, 2003). The increase in PSD-95 may also allow further NMDARs to link with other downstream signalling pathways (Komiyama et al, 2002) thereby facilitating at least some of the plasticity underlying the central sensitisation phenomenon. However, it is interesting to note that one of the proteins associated with one of the downstream signalling pathways, SynGAP, is not significantly expressed in the

spinal cord (Garry and Fleetwood-Walker, unpublished data) and it is not known if it is present in the trigeminal complex.

Interestingly, these experiments show that in the trigeminal system no detectable increase in PSD-95 expression following CCI could be observed in contrast to that seen in the spinal cord. This difference might be due to PSD-95 fulfilling a rather different role under usual circumstances in the trigeminal compared to the spinal cord. One possibility is that PSD-95 in the trigeminal complex might facilitate localisation and functional coupling of NMDARs under usual conditions, whereas in the spinal cord, PSD-95 is only induced to achieve this following nerve injury.

In the mouse, PSD-95 has been shown to be integral to the plasticity of the whisker barrel formation seen in the somatosensory cortex (Skibinska et al, 2001) with levels of the protein rising in areas of the cortex associated with a single whisker following training and selective manipulation of that whisker. PSD-95 is also believed to be vital for the proper formation of the barrel architecture throughout the trigeminal brainstem complex during development, as PSD-95 mutant mice show an incomplete barrel development and maturation (Mark Barnett, University of Edinburgh, unpublished communication). Also, barrel architecture is not as distinct in the oralis region as elsewhere in the trigeminal complex (Bennett-Clarke et al, 1992) and this may explain the apparent lower ratio of PSD-95 to GAPDH in the oralis region compared to the caudalis. Given that from immunoblots, the level of PSD-95 that is expressed relative to the housekeeping protein GAPDH is much higher in the trigeminal complex than in the spinal cord, then PSD-95 may be in significantly greater abundance in the trigeminal system than it is in the spinal cord. It would be beneficial to view this expression directly by Immunohistochemistry. However, the postsynaptic density is a highly insoluble complex and access to antigen is very difficult even with specialist antigen retrieval techniques. Because of this, attempts to show expression of PSD-95 in either normal animals or following CCI by immunohistochemical means have so far been unsuccessful.

If this protein is indeed expressed widely throughout the complex under basal conditions, then either the induction of PSD-95 in this region following CCI may be inconsequential as a large amount would already exist in the area ready for use, or a

small local increase in the outer zones of the complex may be too small an increase to be detected given the sample area.

Although PSD-95 has been shown to affect surface expression of NMDARs through its integral function to NR2B-mediated receptor internalisation, Chapsyn-110/PSD-93 does not (Tao et al, 2003a). It is interesting to note that in another recent study by the same authors (Tao et al, 2003b) a reduction of Chapsyn-110/PSD-93 in the spinal cord caused by antisense treatment resulted in a blunting of pain behavioural responses in animals following both nerve injury and inflammatory insult with complete Freund's adjuvant. The reduction of the same protein in the trigeminal nucleus caudalis did seem to correlate with a longer time taken for development of neuropathic pain behaviours in the trigeminal model compared to the spinal model, but it is not clear whether this plays a causal role.

Cultured adult spinal dorsal horn neurons from Chapsyn-110/PSD-93-null mice showed a decrease in the surface expression of NR2A and NR2B by 64% and 50% respectively, but no difference in total NR2A/B subunit count (Tao et al, 2003a). This indicates the possibility of PSD-93 affecting the synaptic expression of NR2A/B.

It is possible that PSD-95 and Chapsyn-100/PSD-93 may not in fact be recruited in the same way in the trigeminal caudalis or oralis as they are in the spinal cord and that this might affect NMDAR expression at the synapse.

In these experiments we have also found that SAP-97 is clearly and unequivocally expressed in both the spinal cord and trigeminal system as shown by Western blotting. This is contrary to previous studies which were unable to detect SAP-97 in the spinal cord (Tao et al, 2000, 2001). However, no evidence for an injury-induced change could be found for this protein or SAP-102 which was also present.

The involvement of the NMDAR in neuropathic pain states has lead to extensive research into NMDAR-selective antagonists as possible therapeutic agents, and so far, moderate affinity selective NR2B antagonists have had some success in the treatment of spinal neuropathic pain (Parsons, 2001). Park et al, 2001 have shown that the tooth pulp stimulation-induced plasticity in the oralis involves NMDA

receptor-dependant mechanisms, but as NMDARs in this region do not express the NR2B subunit (Watanabe et al 1994), the selective NMDA NR2B subunit antagonists which have been shown to be effective in reducing neuropathic changes in the spinal cord (Boyce et al, 1999) could not be expected to exert specific effects in the oralis. This suggests that in the trigeminal complex the problem of treating neuroplastic changes known to be associated with pain behaviours may be more complicated.

Clearly there is some disparity in the distribution of key proteins thought to be involved in central sensitisation between the spinal cord and trigeminal system and even between areas of the trigeminal system itself. The most striking difference is the complete absence of NR2B expression that is shown in the trigeminal oralis. This finding corroborates that of Watanabe et al's 1994 paper, which found no presence of NR2B mRNA in the oralis or interpolaris, but did find expression in the caudalis. This finding is important because central sensitisation has been demonstrated in both caudalis and the oralis regions, at least to inflammatory agents (Chiang et al, 1998; Park et al, 2001). In the same experiments it has also been shown that this sensitisation is dependant on NMDA receptor-dependant mechanisms. In a separate experiment, it has been shown that the selective NR2B antagonist ifenpronil acting on the NMDAR reduces nociceptive behaviours in rats and rabbits as measured in the spinal cord. Taken together, these experiments suggest that selective NR2B subunit antagonists may reduce the demonstrable central sensitisation in the caudalis subnucleus of the trigeminal complex, but not in the oralis subnucleus. This raises the interesting hypothesis that there are also co-existing different mechanisms of central sensitisation in the trigeminal system. This may be analogous to two forms of monosynaptic facilitation seen in the hippocampus (Nicoll and Malenka, 1995) where an NMDA dependant postsynaptic LTP can be demonstrated alongside that of an NMDA independent presynaptic LTP in mossy fibres.

Indeed, as well as the NMDA dependant central sensitisation present in the trigeminal complex, it has been shown in rats that the application of $P2X_3$ and $P2X_{2/3}$ antagonists in the caudalis inhibit the expression of central sensitisation in oralis in response to mustard oil application to the face. These effects may be mediated by presynaptic P2X receptors since P2X receptors localized at central presynaptic

terminals can be activated by ATP and this activation evokes glutamate release in co-cultured preparations (Gu and MacDermott 1997; MacDermott et al. 1999).

Chapter 7
Summary discussion and conclusion

Neuropathic pain due to nerve injury may occur as a result of peripheral tissue damage due to disease or trauma, or may be a direct result of nerve transection, crushing or constriction. The chronic pain states which develop include spontaneous pain, hyperalgesia and allodynia, and they may persist long after the initial injury has healed (Scadding, 1984).

With the evidence that the clinical presentation and duration of neuropathic symptoms in the spinal and trigeminal systems are dissimilar (Hoffman and Matthews, 1990; Sherman et al, 1984; Pollman, 1981; Reisner, 1981), laboratory findings that suggest regional differences in ectopic discharge (Tal and Devor, 1992) and sympathetic nervous system involvement (Benoliel et al, 2001; Bongenhielm et al, 1999), together with anatomical, biochemical and functional differences between these two regions (Bennett and Dubner, 1983; Watanabe et al, 1994; Park et al, 2001; Chiang et al, 2002), it is possible to predict that there may well be differing mechanisms underlying plastic change at the synapses of these two systems.

A possible candidate for the lasting increase in excitability of damaged afferents is the phenomenon of bistability. This is when a cell has the ability to function at two different resting membrane potentials. The first being its normal potential, and the second potential is when the cell is depolarised and the resting membrane potential is altered to a new less negative level and to stays there for sometime. This means that at the second less negative resting potential the cell is more easily depolarised and therefore more readily excited. This type of mechanism could be involved in propagation in networks of excitatory and inhibitory populations directly in cells in the spinal cord dorsal horn or the trigeminal complex (Goloumb and Ermentrout, 2001).

However a more recent finding has shown that some DRG afferent cells fire repetitively depending on their ability to generate subthreshold membrane potential oscillations. Action potentials are triggered when the amplitude of oscillation sinusoids reaches threshold. Only neurones with oscillations fire repetitively; others may fire at the onset of a depolarizing pulse, but activity is not sustained. Axotomy greatly increases the proportion of neurones with subthreshold oscillations and has

been shown to increase ectopic discharge in neuropathic pain models (Study and Kral, 1996; Amir et al., 1999; Liu et al., 2000a, b).

In this study we have attempted to investigate possible differences between neuropathic sensitisation in spinal and trigeminal neurons using electrophysiological and biochemical techniques. We have also investigated the role of candidate proteins that are predicted to influence the processes of degeneration and regeneration of nerve cells, which are intricately linked to the proper functioning of the cell after nerve injury. Such processes could be involved in the transport of factors from the cell body to the terminals over a period of hours to days that is known to accompany both the acute organisational and longer term genomic changes in neurons (Sigel, 1995; Nicoll and Malenka, 1995) and is necessary for the formation of long term synaptic changes thought to underlie neuropathic pain states.

To model spinal and trigeminal neuropathic pain we used the Bennett and Xie (1988) model of chronic constriction injury and modified versions of it (Vos et al, 1994; Garry et al, 2003), in which chromic gut ligatures were tied loosely around the appropriate nerve so as to barely constrict it. Consistent with previous studies (Attal et al. 1990; Bennett and Xie, 1988; Vos et al, 1994), the animals subsequently developed mechanical and thermal allodynia, as well as mechanical hyperalgesia.

Despite their likely involvement with cytoskeletal function, no evidence could be found for any major role of synuclein proteins in neuropathic sensitisation. Although some minor changes in the development and maintenance of this sensitised state were observed in mutant animals, they were within the range of normal experimental variability and could not be specifically related to a functional role in neuropathic sensitisation. This does not mean that these proteins do not have some subtle part to play in the degenerative and regenerative processes associated with nerve damage, but merely that this role does not seem to affect the overall progression of the neuropathic sensitisation.

However we have shown electrophysiologically that (further to the behavioural sensitivity) chronic constriction injury causes sensitisation in both spinal sciatic and trigeminal nerve injury models, and that the intensity coding to graded mechanical

stimuli neurons in the spinal dorsal horn and trigeminal caudalis of naive animals show distinct differences:

- Spinal and trigeminal neurons show marked differences in mean initial responsiveness between normal and neuropathic animals, and neurons in neuropathic animals show marked post stimulus discharge response (PSDR) properties compared to neurons in naïve animals.
- Trigeminal neurons from neuropathic animals show much greater PSDR activity than spinal neurons from neuropathic animals, and neuropathic recordings can be subdivided to show two different categories of neuronal response.

Furthermore, in response to mechanical brush conditioning stimulus to the face, trigeminal complex neurons from neuropathic animals show a propensity for further excitation in addition to that already induced by nerve damage. The reverse of this is seen in response to a conditioning cold stimulus.

We further investigated whether differential biochemical changes might underlie the differences in spinal/trigeminal sensitisation. We showed in the spinal cord key proteins that are known to contribute to the development and maintenance of central sensitisation are dynamic in their expression and that the characteristics of these changes are different between both the spinal cord and the trigeminal system. Although the assessment by immunoblot is a relatively gross method of looking at NMDA receptor subtype expression in these regions (because we are examining regions of the brainstem tissue rather than specific neuronal populations), the experiments described in this thesis have added further evidence to suggest differences in the mechanisms of neuropathic plasticity between the spinal dorsal horn circuits and those in the trigeminal complex. The involvement of distinct subtypes of NMDA receptor and associated docking proteins in the trigeminal system is only beginning to be addressed. Our experiments have shown that changes in protein expression following nerve damage may differ between the spinal dorsal horn and the trigeminal complex and in fact may not be the same in discrete areas of the trigeminal complex itself.

The dominant model of activity-dependant synaptic plasticity for the past 30 years has been LTP. LTP is induced by a brief high-frequency train of synaptic activity. In contrast, more prolonged low frequency synaptic activity gives rise to a long term depression (LTD), this is also blocked by NMDA receptor antagonists (Bliss and

Collingridge, 1993; Dudek and Bear, 1992). It is thought that whereas high frequency bursts of synaptic activity may produce a large and fast increase in intracellular calcium concentration leading to LTP, low frequency stimulation may produce a more prolonged and moderate change in intracellular Ca^{2+} leading to LTD (Lisman, 1989; Yang et al, 1999). A recent paper (Liu et al, 2004) has shown in hippocampal neurons, using specific antagonists for NR2A- and NR2B-containing NMDA receptors, that LTP is mediated by NR2A-containing NMDA receptors and that LTD by NR2B-containing NMDA receptors, and that this may be a function of the differential time decay constant of synaptic responses these two receptors generate (Cull-Candy et al, 2001). Although the authors point out that this rule in the hippocampus may not be universal, it is tantalising evidence that the ratio of NR2A/NR2B at the synapse may be crucial in the development of synaptic changes.

Also recently (Iwamoto et al, 2004) have demonstrated that PSD-95 exerts differential modulatory effects on NR2A and NR2B NMDA receptors. Using the *Xenopus* oocyte expression system, the authors show that PSD-95 potentiates the channel activity of the NR1-NR2B receptor and reduces its sensitivity to L-glutamate but does not do this for the NR1-NR2A receptor. It also inhibits the potentiation of the channel activity of both NR1-NR2A and NR1-NR2B receptors mediated by protein kinase C (PKC), as well as that of NR1-NR2A channel activity, mediated by the tyrosine kinase Src.

Moreover, it has also been proposed that learning-induced regulation of NMDAR composition is a mechanism for constraining further activity-dependent synaptic enhancements, thereby contributing to long-term maintenance of memory (Quinlan et al, 2004).

The data presented in this thesis show that in the spinal cord there is a demonstrable decrease in NR1 subunit expression and an increase in NR2B subunit expression, and in the trigeminal caudalis there is an increase in the level of NR2B subunit alone. Although as discussed previously, this rise in NR2B subunit expression may not necessarily lead to a direct increase in the concentration of NR2B-NMDA receptors at the synapse (Barria and Malinow, 2002), it may be sufficient for an increase in extra-synaptic NR2B-NMDA receptor concentration.

In the primary afferents NMDA receptors are expressed and are involved in the control of substance P and glutamate release from terminals (Liu et al, 1997). In this paper the authors suggest that presynaptic NMDA receptor activation increases substance P release and may affect glutamate release also as they are co-expressed in

the same small diameter afferent fibres. C-fibre stimulation could evoke more substance P and glutamate release and a build up of glutamate at the synapse may well be enough to overspill and activate extra-synaptic post-synaptic receptors, in this case further NR2B-NMDA receptors, possibly affecting cellular calcium entry. This putative mechanism cannot be true for the subnucleus oralis region as no NR2B is seen expressed at all at this gross level. Taken with the clinical findings that tractotomy of the caudal components of the trigeminal complex is sufficient to undermine plasticity seen after inflammatory insult but does not always alleviate chronic pain (Sessle, 2000), then it is intriguing to postulate what other mechanisms may be involved.

To examine what these differences mean in terms of function, further experiments are necessary. For instance, more accurate knowledge of the location of NMDA receptors and their subtypes in the trigeminal complex is vital. Elucidating the distribution of these receptors between pre and post-synaptic terminals and the relative concentration of various receptor subtypes at these locations will be crucial for our understanding of the dynamic processes underlying neuropathic pain states. Even closer examination of the location of NMDA receptor subtypes at synaptic and extra-synaptic sites may also be useful.

As well as using immunohistochemical methods to investigate the precise location of NMDA receptors and their subunit types, pharmacological studies using subtype selective agonists and antagonists to NMDA receptors in an electrophysiological preparation would allow functional insights into their actions. Unfortunately due to time restraints during this study, these types of pharmacological experiments could not be completed.

In conclusion, the present work has shown important differences in behavioural indices, electrophysiological responsiveness and biochemistry for central neurons in a model of chronic neuropathic pain at spinal and trigeminal levels. This suggests that aspects of sensitisation may be distinctly different in different regions of the CNS and may provide opportunities for selectively manipulating these processes to provide pharmacological agents tailored to trigeminal pain relief.

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0649 Mechanistic Comparisons Between Trigeminal and Spinal Neuropathic Pain

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Objectives: Chronic neuropathic pain is resistant to classical analgesics and is characterised by allodynia, hyperalgesia and spontaneous pain. Pain associated with damage to the trigeminal nerve can be particularly persistent, which may reflect differences in trigeminal and spinal mechanisms of synaptic plasticity. Neuropathic sensitisation occurs in the trigeminal spinal complex and at the first synapses in the dorsal horn of the spinal cord. The NMDA glutamate receptor plays a key role in this process and is known to bind to PSD-95, an adapter protein linking the receptor to a complex of signalling, anchoring and other proteins. The objective of this study is to explore potential differences between spinal and trigeminal mechanisms of sensitisation.

Methods: We have investigated the electrophysiological responsiveness of single neurones to mechanical stimuli, in rodent models of chronic constriction injury of sciatic or trigeminal nerve versus normal controls. These have been directly compared for the first time. We measured expression levels of NR1, NR2A and NR2B NMDA receptor subunits and the protein PSD-95, which are proteins known to interact in the postsynaptic density in trigeminal nuclei and spinal cord.

Results: Marked facilitation of responsiveness in thermal and mechanical behavioural reflexes was seen for both trigeminal and spinal neuropathic models. Electrophysiological experiments indicated a marked increase in mechanical responsiveness of individual neurons to stimulation with von Frey filaments. Biochemical experiments revealed that the changes in PSD-95 expression and its potential interaction with other proteins differed between trigeminal and spinal neuropathic pain models.

Conclusions: This study provides evidence for the existence of mechanistic differences in neuropathic sensitisation between trigeminal and spinal regions. Elucidation of these differences may lead to targets for improved therapeutic treatment of intractable pain states.

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Seq #72 - Orofacial Structure and Sensory-Motor Function
11:00 AM-12:15 PM, Thursday, 26 June 2003 Svenska Massan Exhibition Hall B

Back to the Neuroscience / TMJ Program
Back to the 81st General Session of the International Association for Dental Research (June 25-28, 2003)

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Dorsal horn neurones show increased responsiveness in a model of neuropathic pain.

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Aim of Investigation: To evaluate differences in responsiveness of second order neurones in the dorsal horn of the spinal cord to a natural type mechanical stimulus between normal and neuropathic rats in a chronic constriction injury model.
Methods: Extracellular recordings from cells in the dorsal horn of the spinal cord were examined in both normal rats and rats having undergone chronic constriction injury to the sciatic nerve according to that of Bennett and Xie (Pain 1988; 33: 87-107). A range of mechanical forces was applied to the receptive field of the sciatic nerve with Von Frey filaments and responses compared.
Results: A significant difference in the activity of dorsal horn neurones was found between the treated and untreated groups. Neuropathic animals, as shown by behavioural testing, expressed greater levels of activity than normal animals during and as a result of mechanical stimulus. Furthermore, a profound increase in post-stimulus activity was observed in neuropathic animals whilst in normal animals this was non-existent.
Conclusion: Presented is electrophysiological evidence for a change in the activity of second order neurones in the dorsal horn of the spinal cord following chronic constriction injury in a model of neuropathic pain. Underlying mechanisms for this change may lead to a better understanding of the central sensitisation component in the condition of neuropathic pain.
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